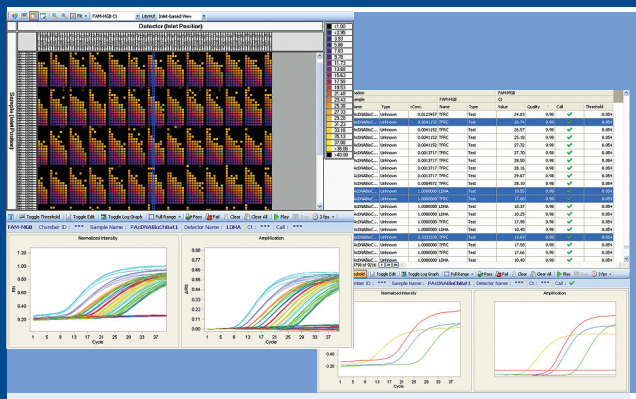
Fluidigm®

USER GUIDE



Real-Time PCR

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About this User Guide

How to Use This Guide

The following chapters provide information about the analysis software and protocols for Real-Time PCR on the BioMark™ or BioMark HD Systems.

Document Conventions

This guide uses specific conventions for presenting information that may require your attention. Please refer to the note conventions below.

CAUTION! This convention highlights potential bodily injury or potential equipment damage upon mishandling of the BioMark System. Read and follow instructions and/or information in a caution note very carefully to avoid any potential hazards.

WARNING! This convention highlights situations that may require your attention. May also indicate correct usage of instrument or software.

IMPORTANT: This convention highlights situations or procedures that are important to the successful outcome of your experiments.

NOTE: This convention highlights useful information.

Related Documents

This document is intended to be used in conjunction with these related documents:

- *Fluidigm® Data Collection Software User Guide* (PN 68000127)
- *Fluidigm IFC Controller Usage Quick Reference* (PN 68000126)
- *Fluidigm 48.48 Fast Real-Time PCR Workflow Quick Reference* (PN 100-2637)
- *Fluidigm 48.48 Real-Time PCR Workflow Quick Reference* (PN 68000089)
- *Fluidigm 96.96 Fast Real-Time PCR Workflow Quick Reference* (PN 100-2638)
- *Fluidigm 96.96 Real-Time PCR Workflow Quick Reference* (PN 68000130)
- *Fluidigm Control Line Fluid Loading Procedure Quick Reference* (PN 68000132)
- *Fluidigm Gene Expression Specific Target Amplification Quick Reference* (PN 68000133)

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Real-Time qPCR

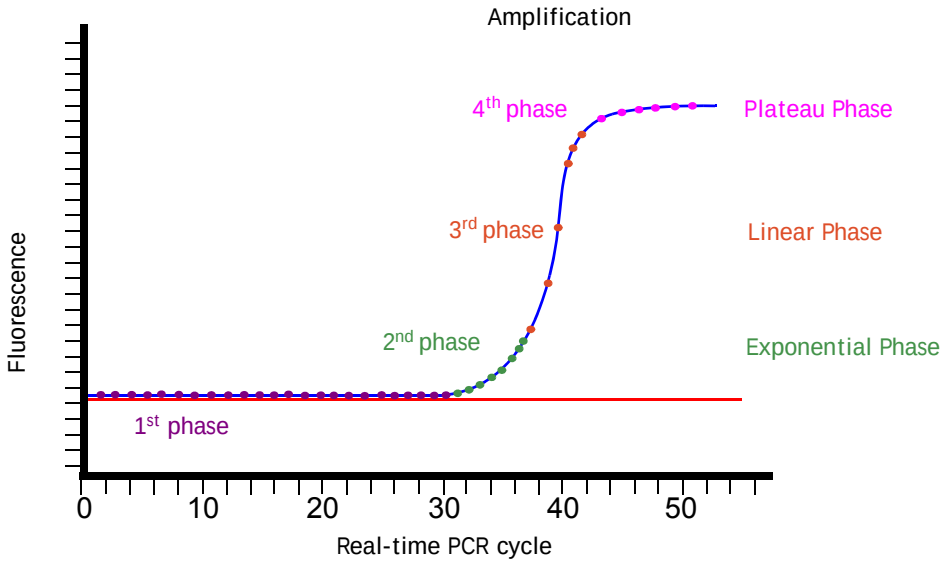
Real-time quantitative PCR (qPCR) is a powerful technique for quantifying changes in gene expression by producing millions of copies of specific, targeted regions of complementary DNA (cDNA) that has been reverse transcribed from messenger RNA (mRNA).

Advantages of Real-Time qPCR

Historically, qPCR has been a time-consuming process because of the time required to get gel-based end-point-measured (plateau phase) results. These results tended to be less accurate, and did not have as wide a dynamic range as real-time PCR. With the advent of quantitative data collection during the exponential phase of PCR, real-time quantification is a reality.

PCR Fundamentals

To appreciate the advantages of real-time PCR, a short review of PCR fundamentals is in order. At the start of a PCR reaction, reagents are in excess, both template and product are at low enough concentrations that product renaturation does not compete with primer binding, and amplification proceeds at a constant, exponential rate. The point at which the reaction rate ceases to be exponential and enters a linear phase of amplification is variable, and, at the plateau phase, the amplification rate drops to near zero.



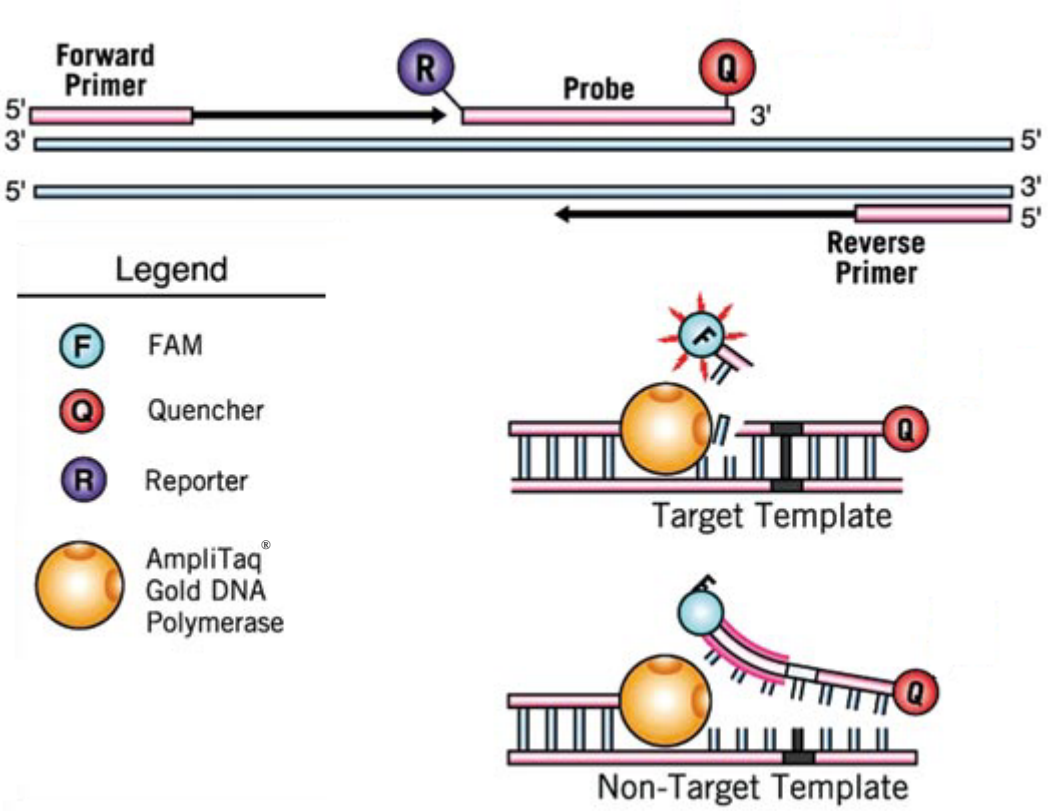
The Exponential Phase

To ensure accuracy and precision, quantitative data is best when collected at a point in which every reaction is in the exponential phase of amplification—this being the only phase in which amplification is easily reproducible.

Advantages of Real-Time qPCR TaqMan® Chemistry

The BioMark HD System uses dual-labeled probes, such as TaqMan® probes, for real-time qPCR amplification.

Dual-labeled probes are oligonucleotides that contain a fluorescent reporter dye on the 5' base, and a quencher located on the 3' base. When irradiated, the excited fluorescent reporter dye transfers energy to the nearby quencher molecule rather than fluorescing, resulting in a non-fluorescent substrate. Dual-labeled probes are designed to hybridize to a complementary region of the cDNA. The probe is flanked by an upstream and downstream primer pair that generates a PCR product. During PCR, when the polymerase extends the PCR product from the upstream primer, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent quencher and reporter dyes and Fluorescence Resonance Energy Transfer (FRET) no longer occurs. The increase in fluorescence intensity is proportional to the number of probe molecules that are cleaved.



BioMark HD System for Genetic Analysis

The BioMark HD System includes the optical, thermal cycling, and software components necessary to perform real-time quantitative PCR (qPCR) analysis on Dynamic Array™ IFCs.

The BioMark HD System provides orders of magnitude higher throughput for real-time qPCR compared to conventional platforms due to its Dynamic Array IFCs — nanofluidic chips that contain fluidic networks that automatically combine sets of samples with sets of assays. This innovative solution for real-time qPCR provides reaction densities far beyond what is possible with microtiter plates and significantly reduces the number of liquid-handling steps and the volume per reaction.

High-End Detection Optics

The BioMark HD System includes a high-resolution CCD camera that covers 30mm by 30mm, an area sufficiently large to simultaneously image all reactions in Dynamic Array™ IFCs. The BioMark HD System optics and analysis software is available for different applications, which are compatible with a variety of Fluidigm chip families for TaqMan® chemistry. The System's computer-controlled chip tray automatically loads the chip into the instrument for ease of use. A barcode reader tracks experiments, reducing the chance of errors.

The BioMark HD System Components

The BioMark HD System includes an internal thermal cycler, flat panel monitor, keyboard, and mouse

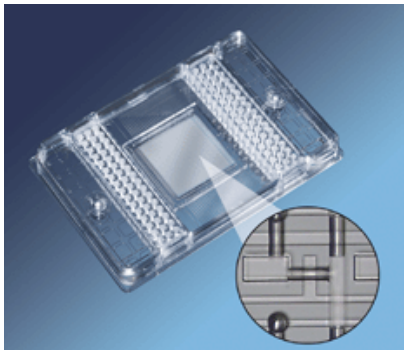
The IFC Controller (single-bay). The MX model primes and loads 48.48 chips while the HX model primes and loads the 96.96 chips.

Dynamic Array IFC Components

Although chip architecture varies, the essential components common to all are highlighted in the graphic below. For more information see the appendices.

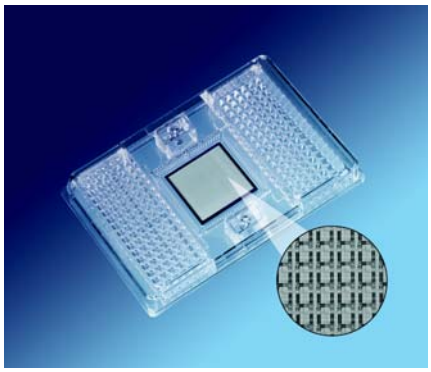
48.48 Dynamic Array IFC for Real-Time Quantitative PCR

The 48.48 Dynamic Array IFC is a matrix of channels, chambers, and integrated valves finely patterned into layers of silicone. Valves within the array partition 48 samples and 48 TaqMan® assays, and allow them to be systematically combined into 2,304 reactions.



96.96 Dynamic Array IFC for Real-Time Quantitative PCR

The 96.96 Dynamic Array IFC is a matrix of channels, chambers, and integrated valves finely patterned into layers of silicone. Valves within the array partition 96 samples and 96 TaqMan® assays, and allow them to be systematically combined into 9,216 reactions.



The following table illustrates the advantages of Dynamic Array IFCs, compared to microwell plates, using TaqMan® assays.

Dynamic Array IFC Features	Dynamic Array IFC Benefits
Lower running costs. Nanoliter reaction volumes	Saves on reagents and pipette tips, and on the upkeep of liquid-handling robots.
Higher throughput. High-density reaction chambers	The 48.48 Dynamic Array IFC provides 2,304 data points per run. The 96.96 Dynamic Array™ IFC provides 9,216 data points per run.
More Informative. An N x M (rows by columns) architecture coupled with nanoliter reaction volumes	Generates multiple readouts per sample, without the spectral overlap and cross-amplicon influence of multiplexed PCRs.
Highly Flexible. Input frame with microwells together with an N x M architecture	Facilitates the input of any set of samples and any set of primers/probes (detectors), delivering the throughput of a fixed array.

After pipetting samples and reagents into microwells on the chip frame, the chip is placed into the IFC Controller. You use a laptop computer and a software user-interface to control the valves and pressure-load the sample and reagent fluids. Samples and reagents are automatically routed to their respective chambers for PCR amplification.

BioMark HD System Process Overview

The simplicity of running experiments on either BioMark HD System is illustrated in the five-step process below. For more information see *Fluidigm 48.48 Real-Time PCR Workflow Quick Reference* (PN 68000089) and *Fluidigm 96.96 Real-Time PCR Workflow Quick Reference* (PN 68000130).

1. Prime the chip.
2. Add the samples and assays to the chip.
3. Load and mix samples and assays.
4. Run your real-time experiment on the BioMark HD System.

Before You Begin

To ensure good experimental results, follow the guidelines listed below.

Organizing Your Work

- Label all reagent and reaction tubes.
- Maintain a separate DNA-free laminar flow hood—do not use nucleic acid samples in this hood.
- Use dedicated pipettes, tubes, and gloves for all manipulations that *do not involve* nucleic acid samples, which never leave the DNA-free ("Sample") laminar flow hood.

Preventing Contamination

- Manipulate DNA samples under a dedicated laminar flow hood (name it, "Sample," for example).
- Use separate dedicated pipettes, tubes, and gloves for all manipulations involving nucleic acid samples, which never leave the DNA-dedicated laminar flow hood.
- Change gloves frequently.
- Use aerosol-resistant disposable pipette tips. Discard tips after each use.
- Use disposable, UV-irradiated plastic ware.
- Ensure that all equipment, including paper, pens, and lab coats are dedicated for use only in a particular laboratory. For example, dedicated laboratory coats for each of the PCR rooms.
- Do not bring contaminated workbooks into clean PCR areas.
- Aliquot PCR reagents.
- Wipe PCR hoods daily with DNA*Zap*™ (Ambion) or a similar DNA decontaminate.
- Use ultra-violet radiation to complete decontamination.
- Ensure that only authorized users work in PCR areas and handle PCR equipment.

- Prepare reagents in a dedicated DNA-free laminar flow hood. DNA-free areas prohibit *any biological material*, including DNA or RNA extracts, and PCR products. Also, in the DNA-free area, prepare and aliquot reagent stocks and reaction mixes.

Handling Nucleic Acid, PCR Mixes, and PCR Reactions

- Prevent carry-over of amplified DNA sequences by setting up PCR reactions in a dedicated laminar flow hood, while keeping post-PCR manipulations separate.
- Add extracted DNA to the PCR reaction mixes in the DNA-dedicated ("Sample") laminar flow hood. Be sure to prepare the PCR reaction mixes in the DNA-free laminar flow hood.
- Keep the amplification room—where PCR machines are housed—separate from the room in which PCR reactions are *assembled* (DNA free laminar flow hood).

Using Controls

- Include—whenever possible—a positive control that amplifies weakly but consistently. Using a strongly positive control sample may result in excess amplified product which may serve as a source of contamination.
- Use well-characterized negative samples such as lambda DNA.
- Include reagent controls containing all the necessary reagent components but excluding test DNA.
- Use decontaminating enzymes such as uracil N-glycosylase (UNG) or Uracil-DNA Glycosylase (UDG) to further minimize the likelihood of contamination.

What You Need for Experiments

This section describes the materials that you need to perform your experiments including reagents we support and sample requirements. In addition, you need the following:

- BioMark HD System
- IFC Controller
- 48.48 Dynamic Array™ IFC **or** 96.96 Dynamic Array™ IFC
- 20X GE Sample Loading Reagent (Fluidigm PN 85000735)—store at 4ºC.
- 2X Assay Loading Reagent (Fluidigm PN 85000736)—store at 4ºC.
- Deionized DNA-free, DNase-free, RNase-free water—store at room temperature.
- DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)—store at room temperature.
- Sample Mix
- Prime/probes sets
- Samples of interest

Supported Detection Reagents

We support the following detection reagents[1] with the BioMark HD System.

Probe Types

- FAM-MGB
- VIC-MGB
- FAM-TAMRA
- FAM-non fluorescent quencher[2]

Additional Probe Types

Fluidigm does not support other probe types at this time, however, additional probe types may be run with the BioMark HD System using the following guidelines:

Fluorophores With...		
Excitation Wavelengths		Emission Wavelengths
between 465 and 505 nm	And	between 500 and 550 nm
between 510 and 550 nm	And	between 540 and 600 nm

PCR Master Mixes

The protocol described in this manual uses TaqMan® Universal PCR Master Mix (2X) (Applied Biosystems, PN 4304437). If you choose to use master mixes other than TaqMan® Universal PCR Master Mix, you may have to alter the protocol described in this manual. Contact Fluidigm Technical Support for additional information.

IMPORTANT: You must use a passive reference.

1. Fluidigm recommends that you only use TaqMan® probes and/or other licensed PCR assay reagents from authorized sources. If you have any questions regarding whether you have a license to use particular reagents in PCR systems, you should contact the appropriate licensor and obtain clarification and their permission if necessary. For example, certain probes and their use may be covered by one or more patents held by Applied Biosystems and/or Roche Molecular Systems, which may be contacted at the Director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404 or the Licensing Department, Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.
2. Contact Fluidigm technical support to discuss your non-fluorescent quencher requirements.

Sample Requirements

DNA Quality

Your cDNA should have an 260:280 Ratio between 1.5 and 1.8. Prior to use on a chip, monitor the integrity of your cDNA on a system such as the Agilent® 2100 bioanalyzer.

cDNA Input

The exact amount of cDNA to be used for each experiment depends on the relative abundance of the target gene. Unless you have concentrations in excess of 1,000 copies of your target template per µl of sample, we recommend that you increase the your target concentration by using specific target amplification as described in *Fluidigm Specific Target Amplification Quick Reference Card* (PN 68000133).

cDNA Storage

Avoid multiple freeze-thaw cycles by storing cDNA at 4ºC. For longer storage, aliquots may be stored at -20ºC.

Reagent Storage

Consult manufacturers' product inserts for storing specific reagents.

Using Real-Time PCR Analysis Software

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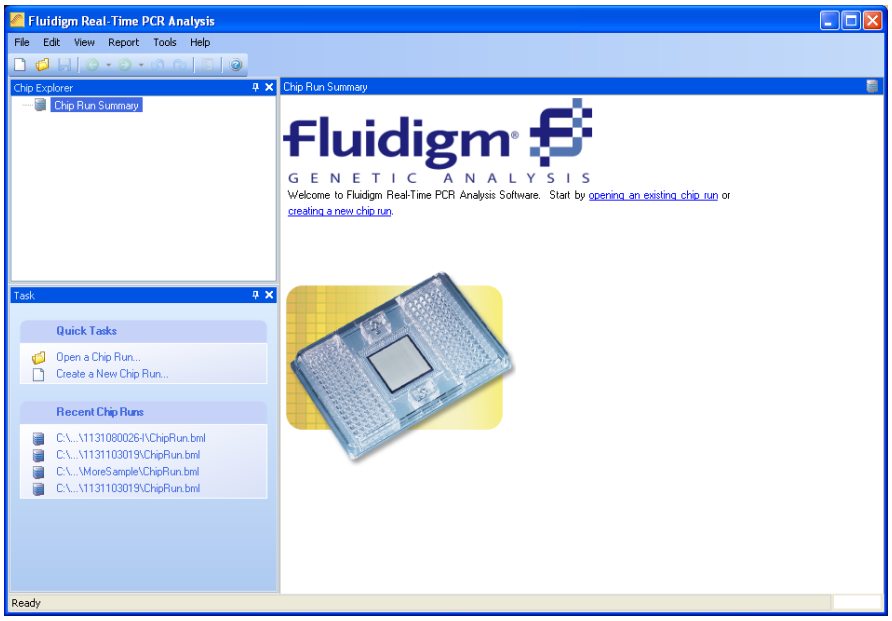
Launching the Software

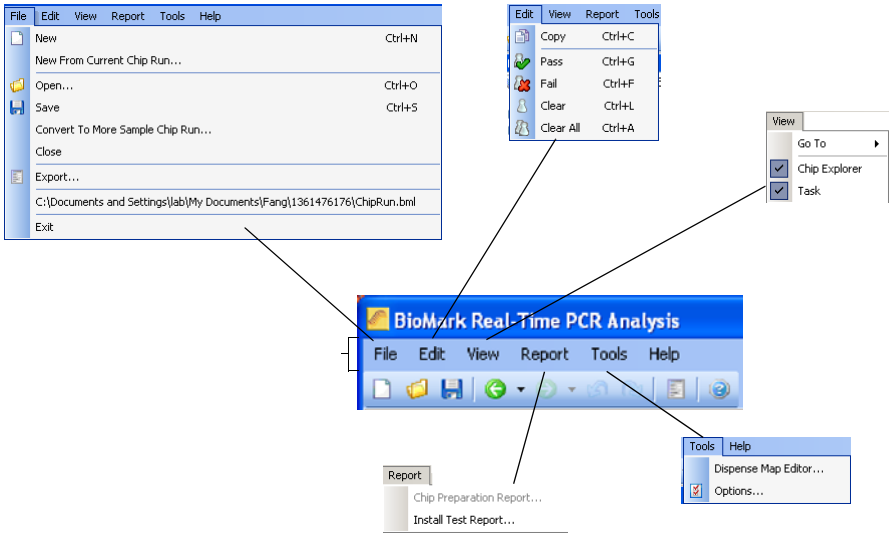
1 Double-click the **Real-Time PCR Analysis Software** icon on your desktop.

The Start screen opens.

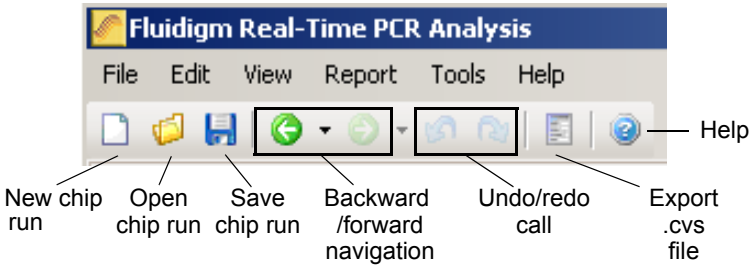


Menus and Icons

Top Menu Bar



Secondary Menu Bar



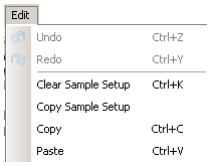
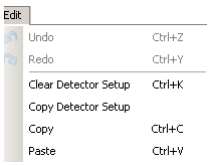
File

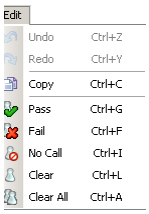
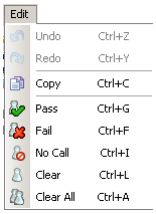
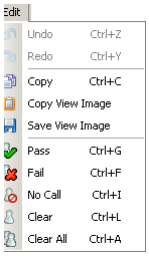
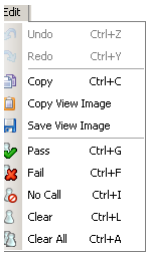
The **File** menu has the following options:

New	Open the **Chip Run Setup Wizard**
Open	Open location of .bml chip run data files
Save	Save your current run data file with any changes
Convert to More Samples Chip Run...	Convert your chip run to a more samples chip run
Close	Close your current run data file
Export	Export **Results** table data or **Heat Map** data as .csv text file
C:\...	Open the location of recently viewed/used .bml files
Exit	Close the application

Edit

The Edit menu options depend on the active window.

If the Active Window is...	Your Options Are...
Sample Setup	
Detector Setup	

If the Active Window is...	Your Options Are...
Analysis Views	
Results Table	
Image View	
Heat Map	

View

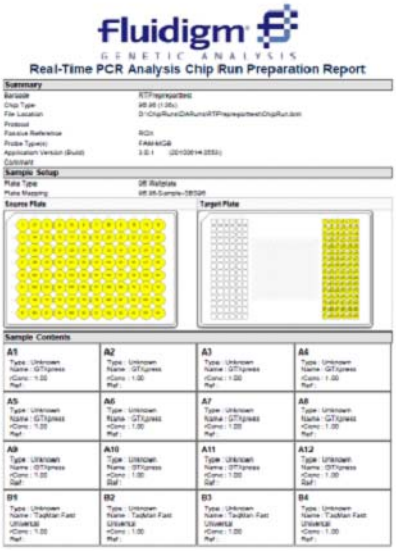
Select **Chip Explorer** and/or **Task** to display these panes in your window.

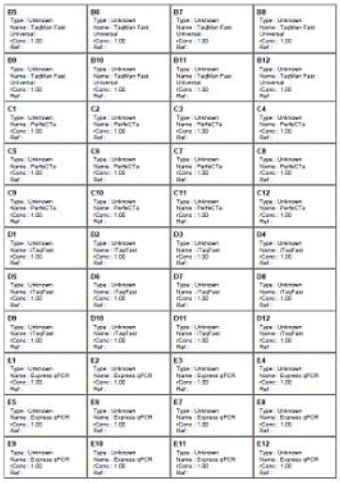
Report

Two reports are available:

- **Chip Preparation Report**

This report records the loading pattern for a chip run. After creating a new chip run file, use the **Chip Preparation Report** to record the data for hand-pipetting.





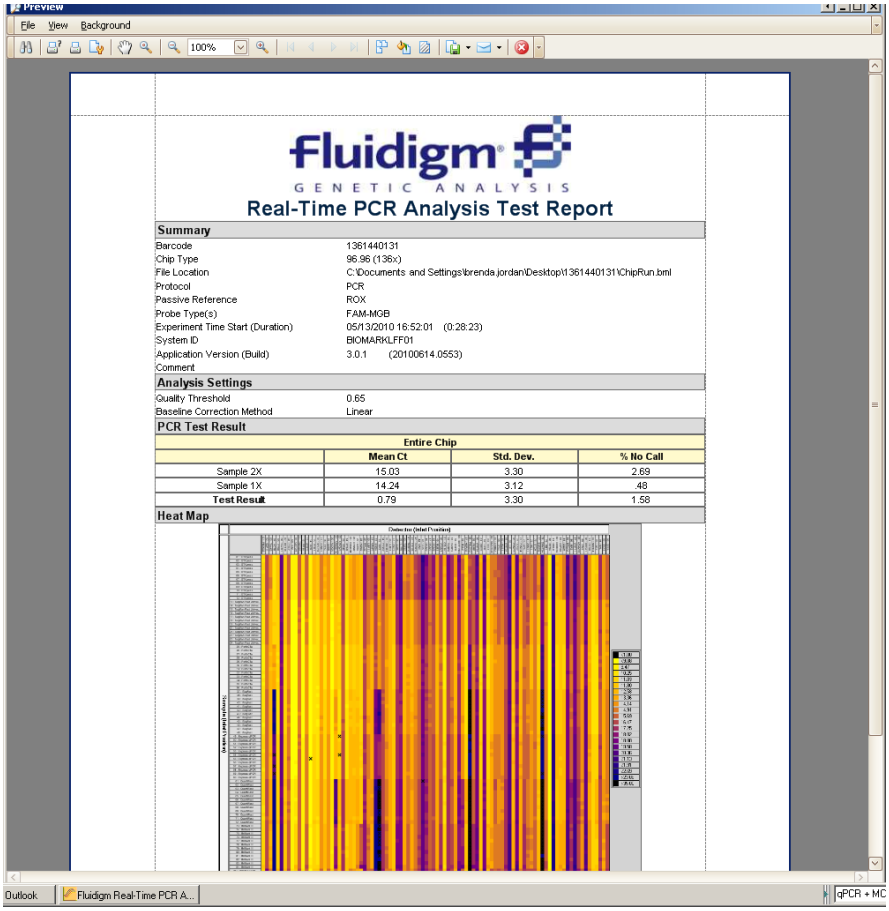
- **Install Test Report**

This report is only available after a chip run (.bml) file has been opened and analyzed in the software.

Install Test Report

1 Select **Report** > **Install Test Report**.

A warning about properly setting up the chip appears. Click **OK** if the setup is correct. The report is generated.



2 Go to **File > Export Document**.... to select a file format. PDF is the default.

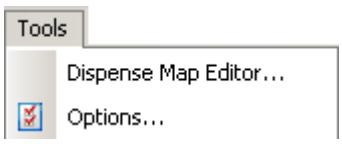
3 Select a folder location to save the file.

4 Change the Name and/or file type, if needed.

5 Click **Save**.

Tools

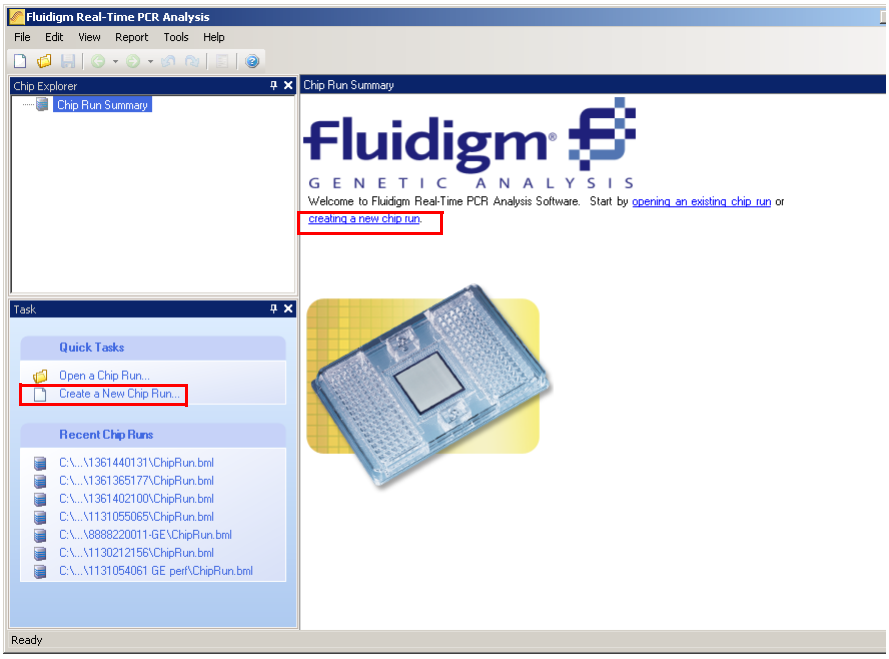
The Tools menu has the following options:



See "Using the Dispense Map Editor" on page 40.

Creating a New Chip Run

1 Click **creating a new chip run** under **Chip Run Summary** or click **Create a New Chip Run** under **Task**.



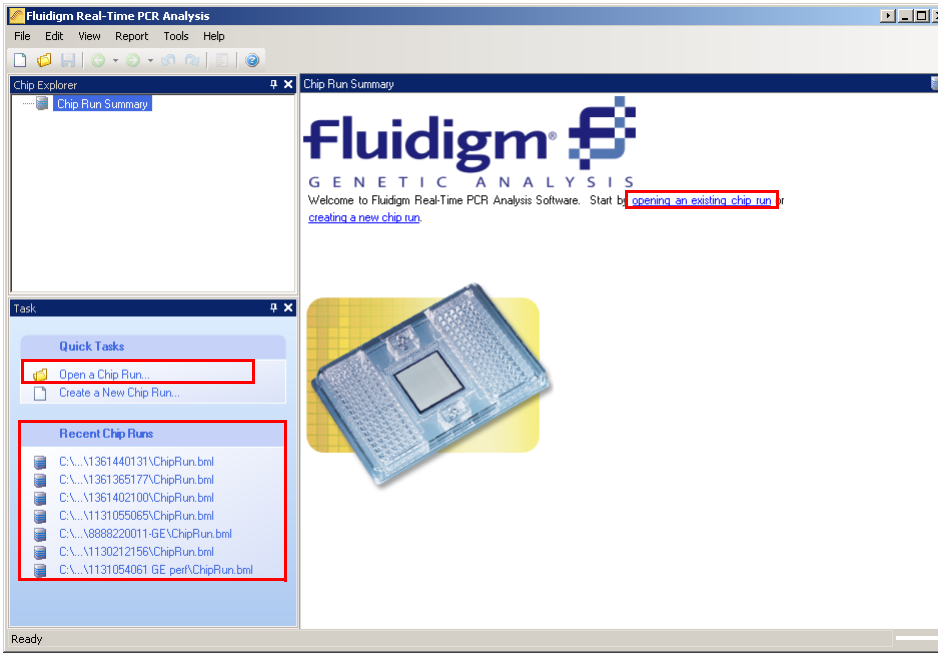
The **Chip Run Setup Wizard** opens.

2 Follow the steps to complete the setup.

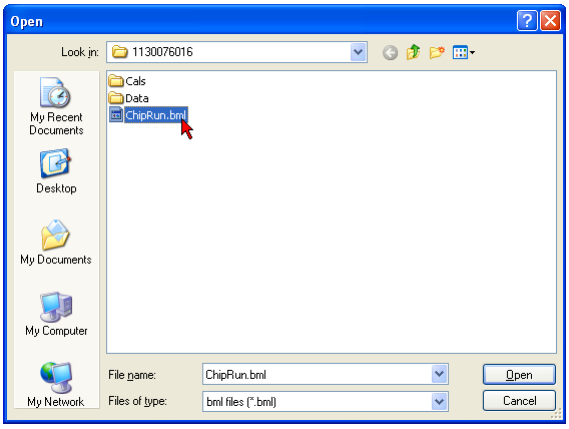
3 Complete the wizard and go to "Setting Up a Sample Plate" on page 33.

Opening an Existing Chip Run

1 To reanalysize a previous chip run, click **open an existing chip run** under **Chip Run Summary** or click **Open a Chip Run** under **Task** or click **File > Open**.

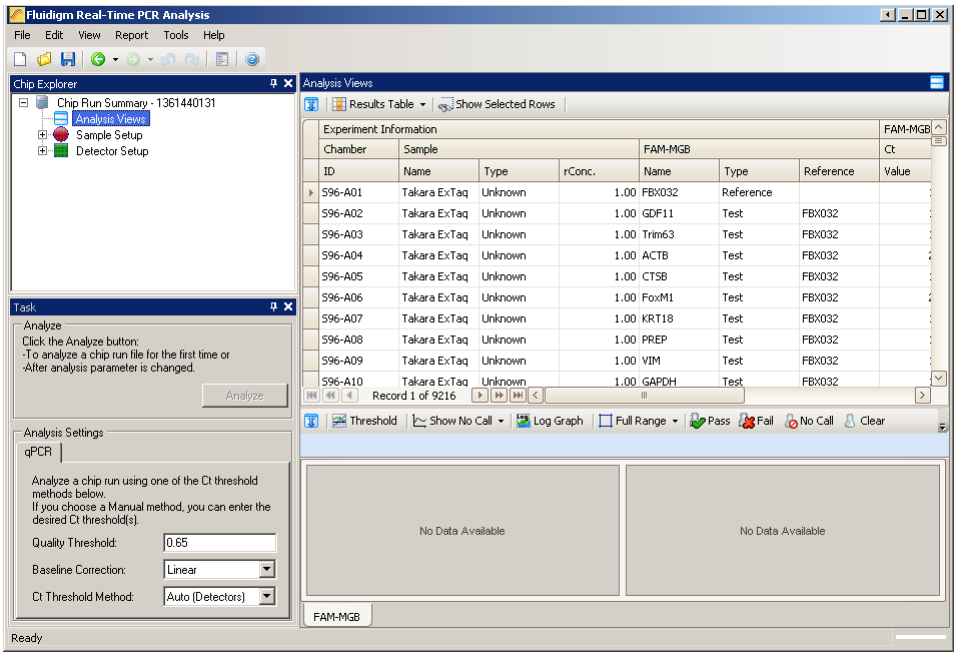


2 Double-click the chip run file (.bml extension).



The chip run file opens.

3 Click **Analysis Views**.



4 Click **Analyze**.

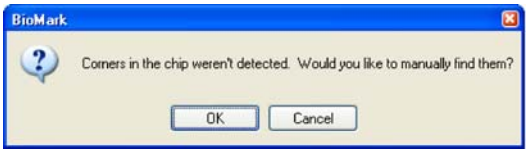
IMPORTANT: You must click **Analyze** each time you change parameters. A reminder dialog appears if you do not click **Analyze** after each change.

The first time a chip is analyzed, the chamber-finding algorithm locates the chamber boundaries of each captured image. This may take some time.

Continue with "Setting Up a Sample Plate" on page 33.

Finding Corners Manually

During the first analysis, if the chamber finding algorithm cannot locate the four corner cells of the chip, an error message appears, asking if you would like to manually find the corners.

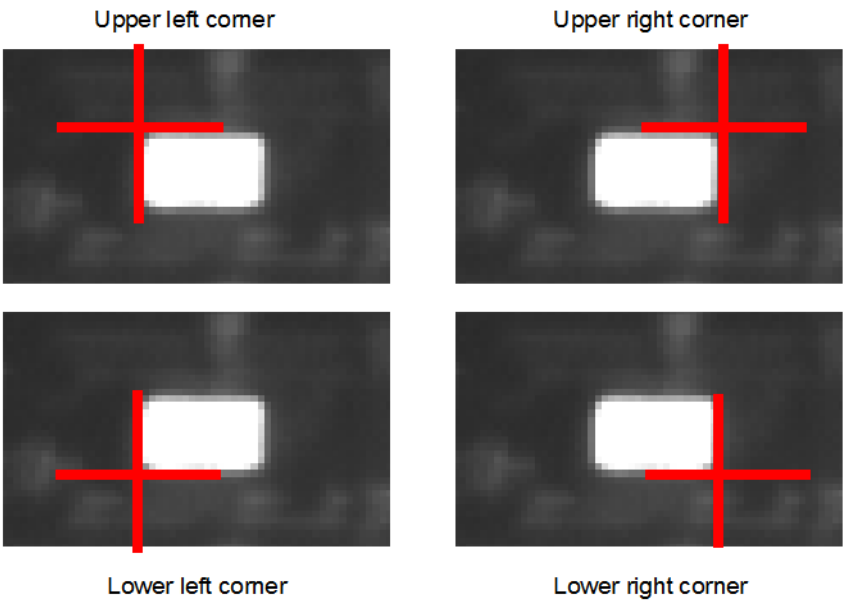


To manually set the corners and analyze the chip:

1 In the error message, click **OK**.

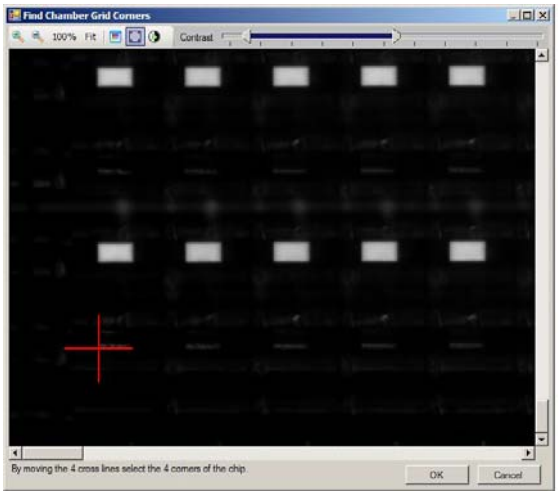
2 Zoom in to see the corner cells.

3 Move the red cross hairs to each of the four corner cells. Make sure each cross hair is on the outer edges of each corner cell.



4 Click **Done**.

NOTE: If little or no ROX is present, the corner cells are very dark. If you cannot see the four corner cells, try adjusting the Contrast slider. You may have to count the number of rows and columns (48 down, 48 across) to make sure you are placing cross hairs correctly.



Forcing a Manual Corner Find

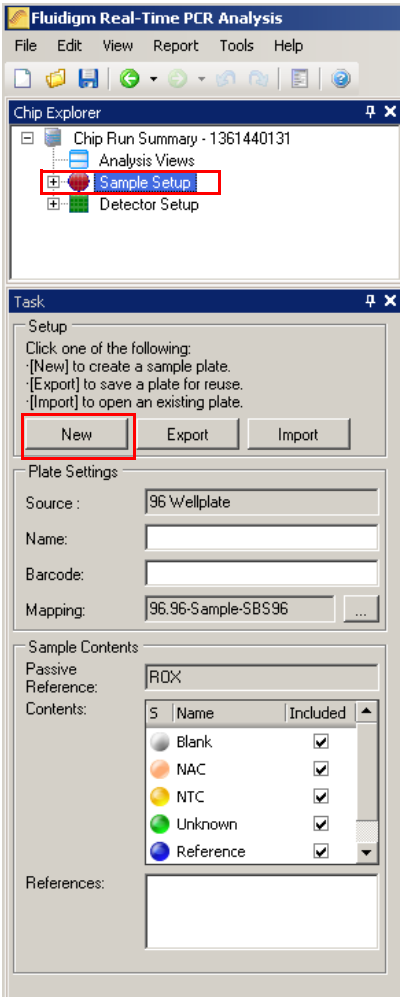
If an automated manual corner find is not satisfactory, you can force a manual corner find by pressing **CTRL** and simultaneously clicking **Analyze**.

Setting Up a Sample Plate

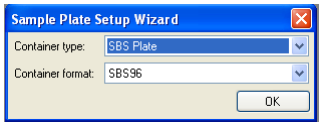
Use this table as a guide when annotating your samples:

Sample Name	Description
Blank	An unused position. Nothing in the chamber.
NAC	**No Amplification Control:** usually the Taq polymerase is left out of the reaction; this is a negative control confirming that positives cannot occur without the PCR working.
NTC	**No Template Control (negative control)**: everything included except the sample; to show that a positive result cannot be obtained when the sample is left out.
Unknown	An experimental sample.
Reference	A sample against which the unknown samples are compared or normalized.
Standard	A sample against which unknown samples are compared in a standard curve analysis.

1 In the Chip Explorer window, select **Sample Setup**.



The **Sample Plate Setup Wizard** opens.



1 Choose the appropriate **Container type** and **Container format**.

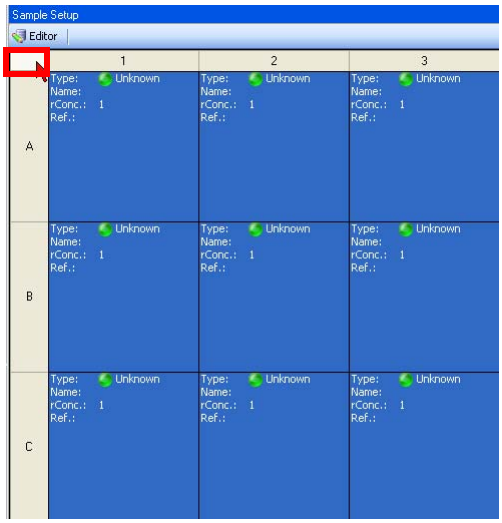
Container type	• **SBS Plate:** the plates where samples and detectors are stored before being pipetted into chip. • **Sample Inlets**: location where samples enter the chip.
Container format	• SBS96: a 96-well plate.

2 Click **OK**.

3 Select the cells to use as a reference.

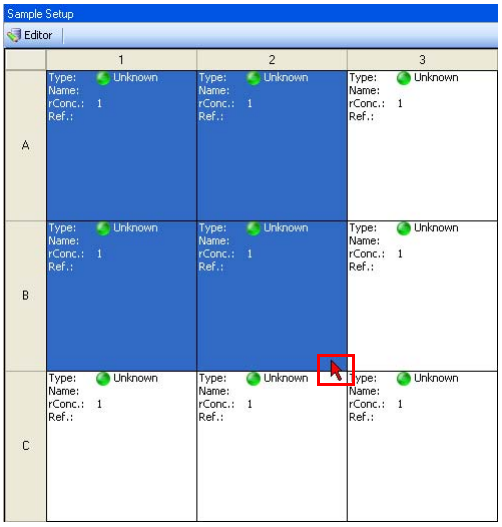
- Click the upper left corner to select all the cells.
- Click and hold while dragging your cursor through cells.

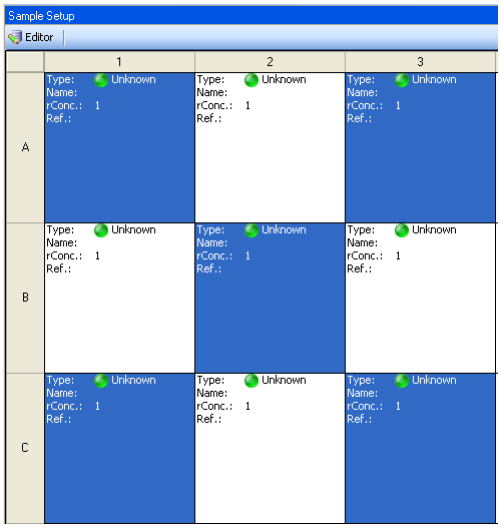
- Click individual cells while pressing the **CTRL** key.



Click the upper left corner to select all cells

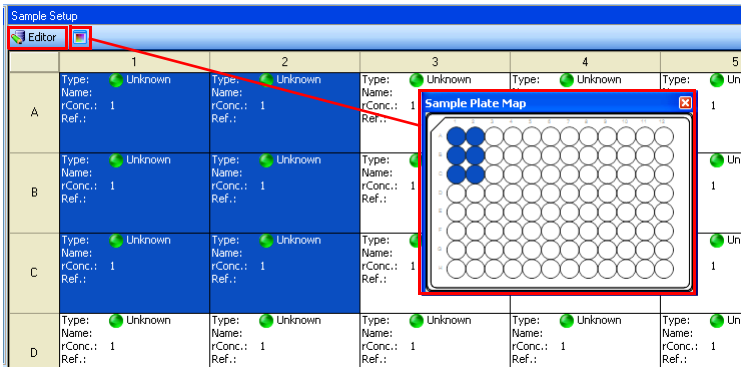
Click and drag to select





Press and hold **CTRL** while clicking individual cells

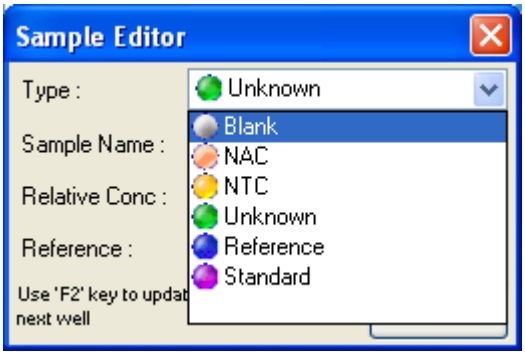
4 OPTIONAL: Click the **Sample Plate Map** icon .
The map shows selected cell(s) relative to the entire sample plate.



5 Click **Editor**. The **Sample Editor** opens.

6 Select the appropriate type.

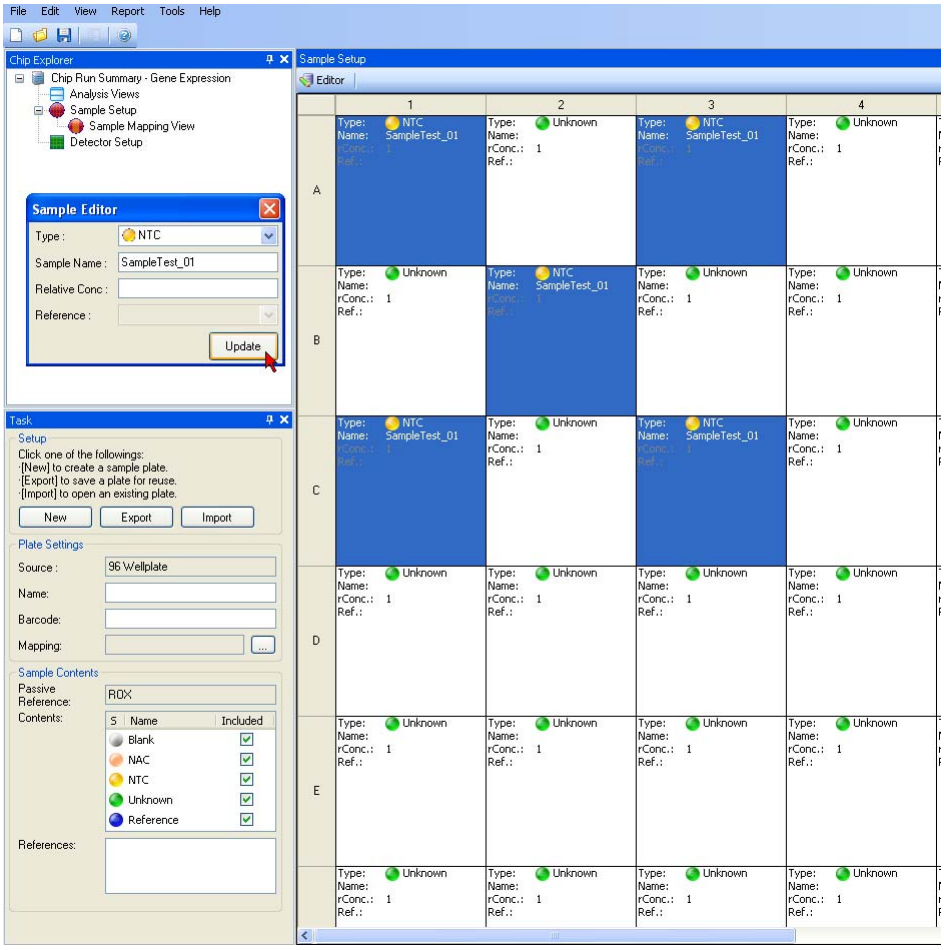
NOTE: To identify a reference, see "Calculating Delta Ct Sample Values" on page 105.



7 Enter the sample name.

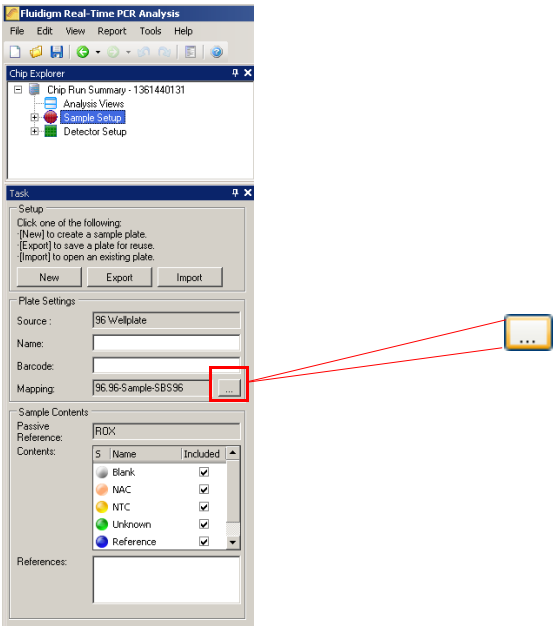
8 Enter the relative concentration.

9 Click **Update**. The **Sample Plate Setup** reflects the updates.



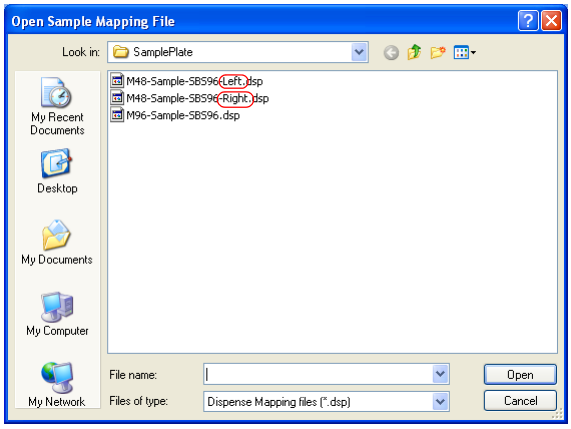
10 Close the **Sample Editor**.

11 Click the **Open Mapping File** icon.

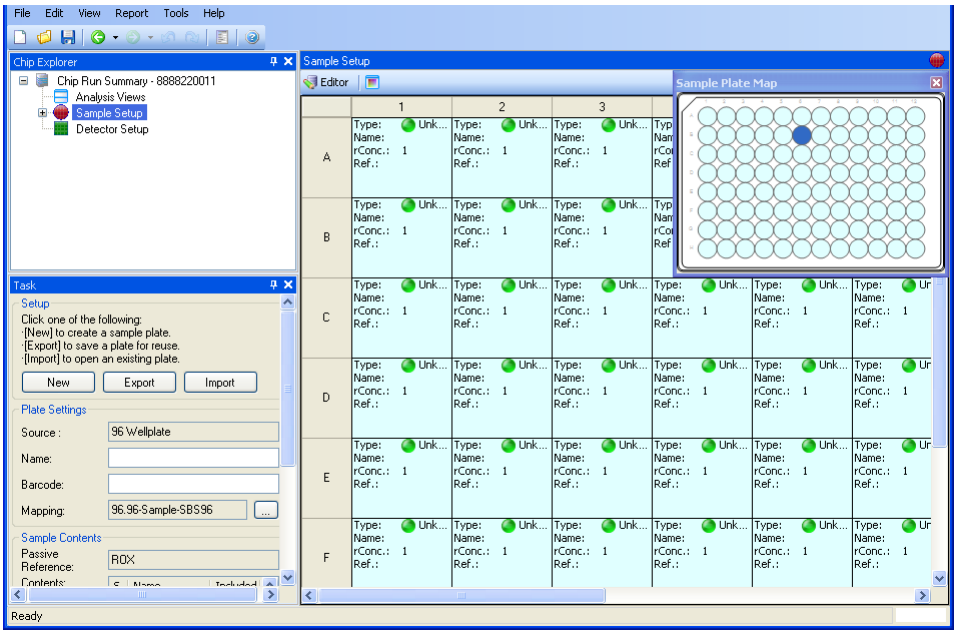


12 Double-click either left or right sample mapping file to determine dispense location.

NOTE: If you are analyzing a 96.96 chip, select **M96-Sample-SBS96.dsp**.



Your selection is displayed in light blue (left or right).

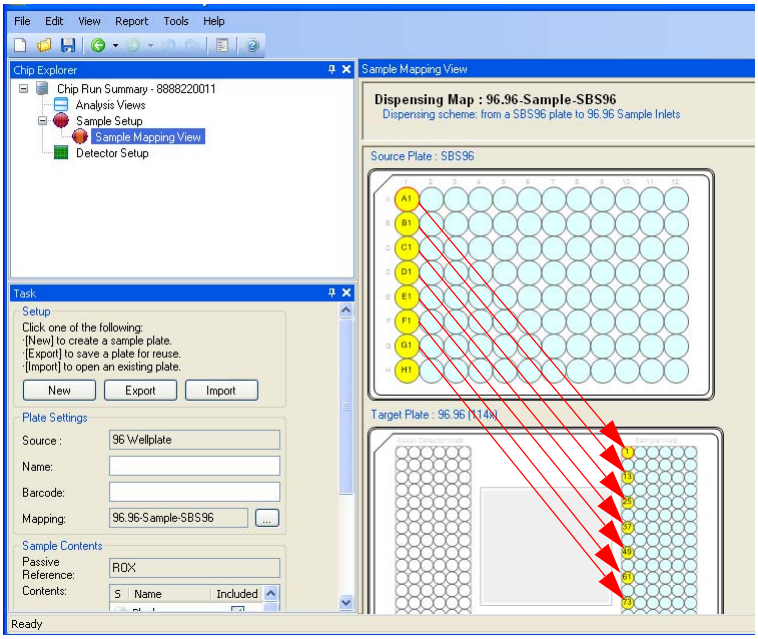


Your sample plate setup is complete. Next, go to "Using the Sample Mapping Viewer,"

Using the Sample Mapping Viewer

Use the **Sample Mapping Viewer** to view or record the loading pattern after setting up the sample plate.

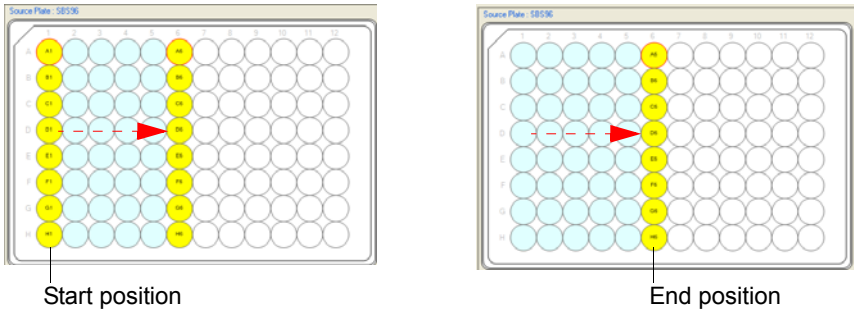
1. Click **Sample Mapping View**. The dispensing map opens.
2. Click a cell in the **Source Plate** to see where it loads on the **Target Plate**.

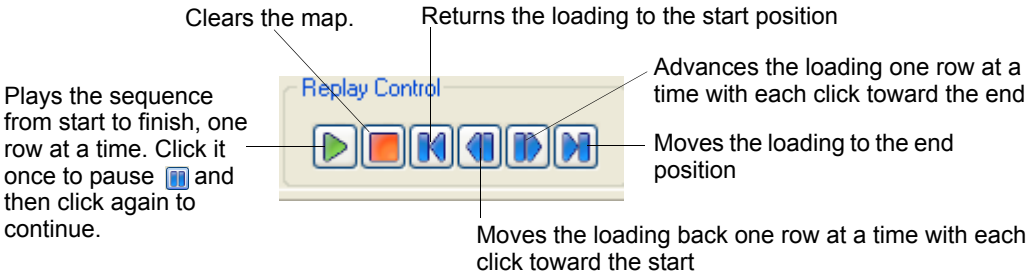


NOTE: If you click an unused cell, the “Well not used” warning appears.

Using the Replay Control

Use the **Replay Control** to show where and in what sequence the **Target Plate** receives the samples from the **Source Plate**.

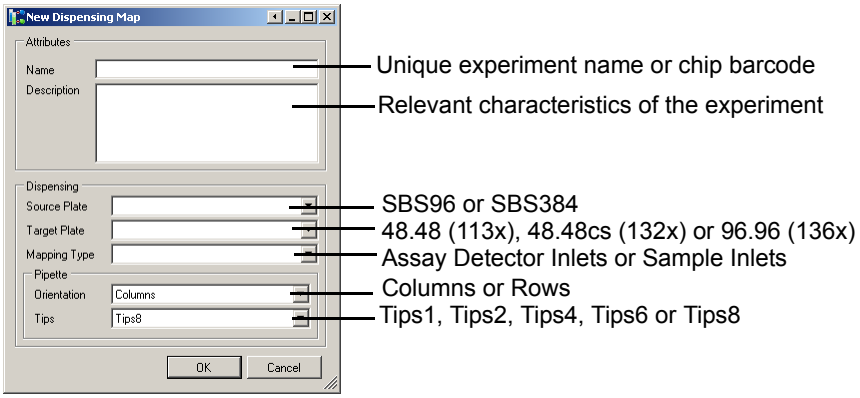



Clears the map.
Returns the loading to the start position
Plays the sequence from start to finish, one row at a time. Click it once to pause and then click again to continue.
Replay Control
Advances the loading one row at a time with each click toward the end
Moves the loading to the end position
Moves the loading back one row at a time with each click toward the start

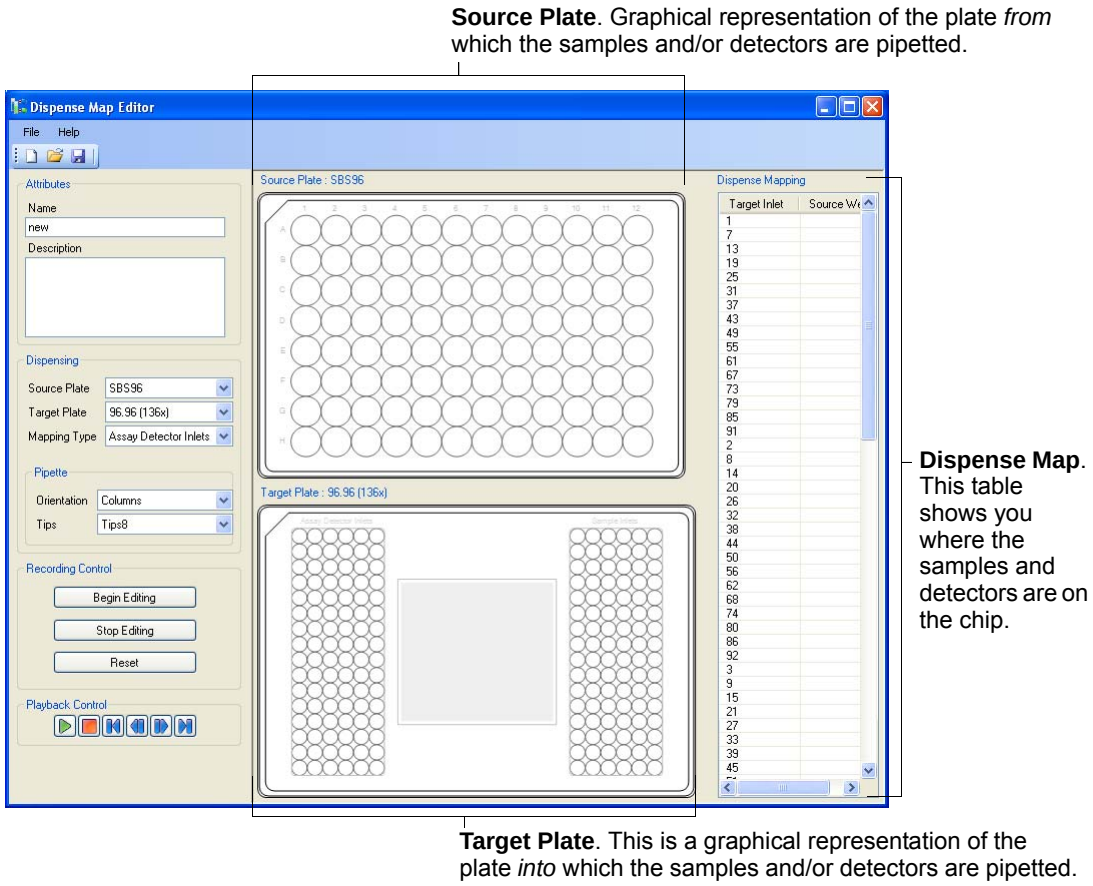
Using the Dispense Map Editor

Use the **Dispense Map Editor** to record custom load maps for future use. After recording your loading sequence, you can save it and play it back anytime.

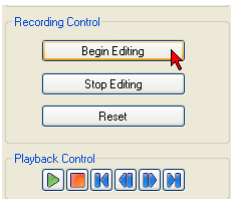
1. Click **Tools > Dispense Map Editor**.
2. Click **New**. The **New Dispense Map** window opens.
3. Complete the **New Dispense Map** using the following as a guide.



4 Click **OK** to open the new dispense map in the **Dispense Map Editor**.



5 Click **Begin Editing** in the recording control pane.



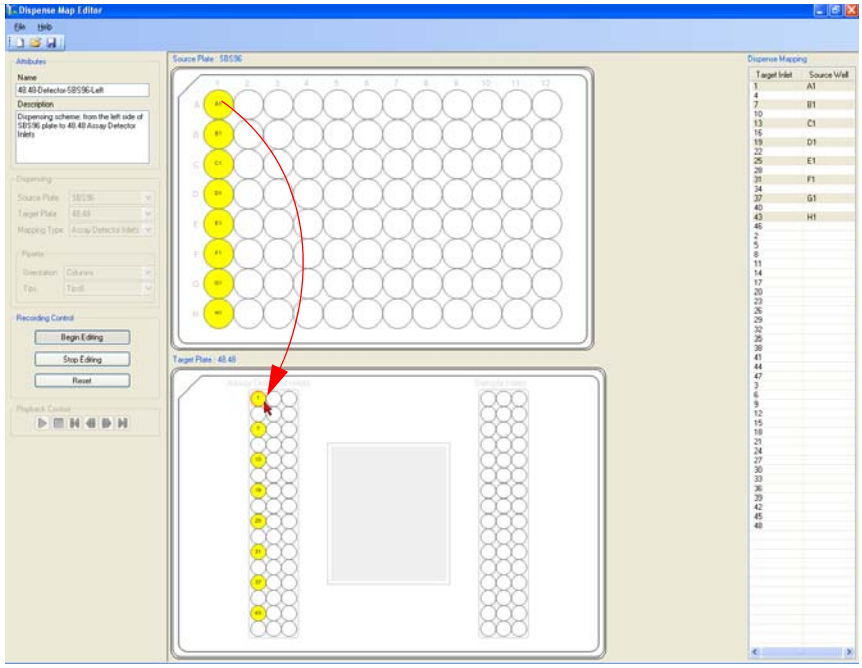
a Click the first cell from the **Source Plate**. Then, click the location in the **Target Plate**.

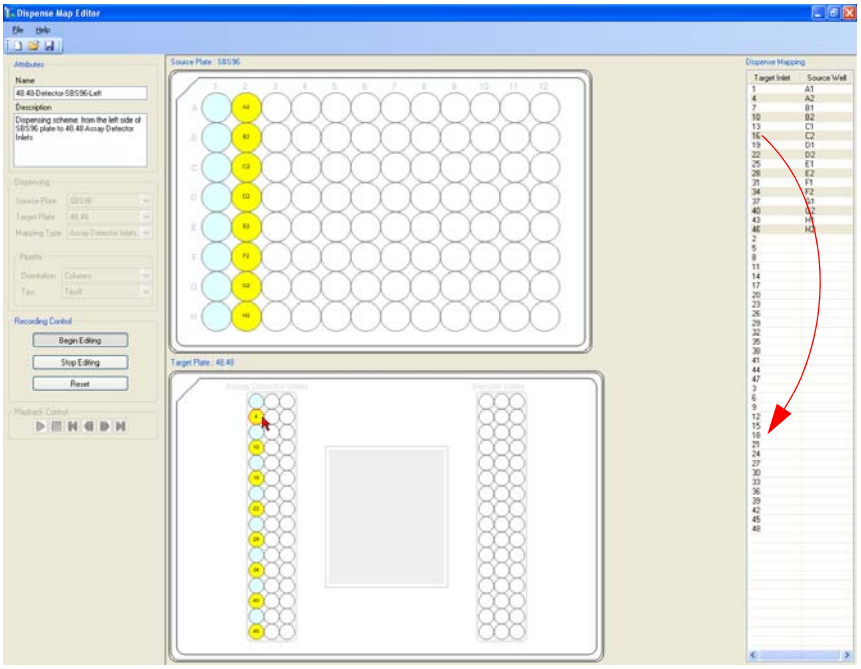
b Continue clicking appropriate cells (from the **Source Plate** to the **Target Plate**) until your custom loading map has been recorded.

NOTE: When you click **Begin Editing**, the dispensing pane becomes inactive.

6 Click **Stop Editing**.

These graphics show custom loading and how it looks as you proceed.





7 Review the loading pattern you have recorded by clicking the green arrow button in the playback control pane.

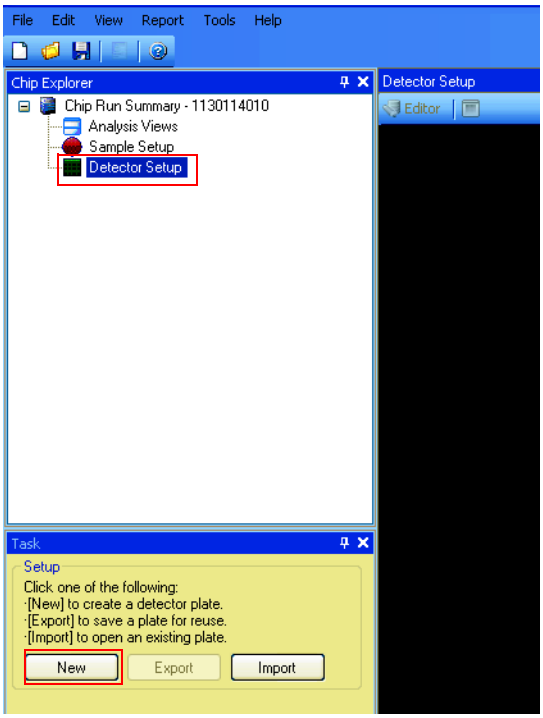
Setting up a Detector (Assay) Plate

Use this table as a guide when annotating detectors (assays):

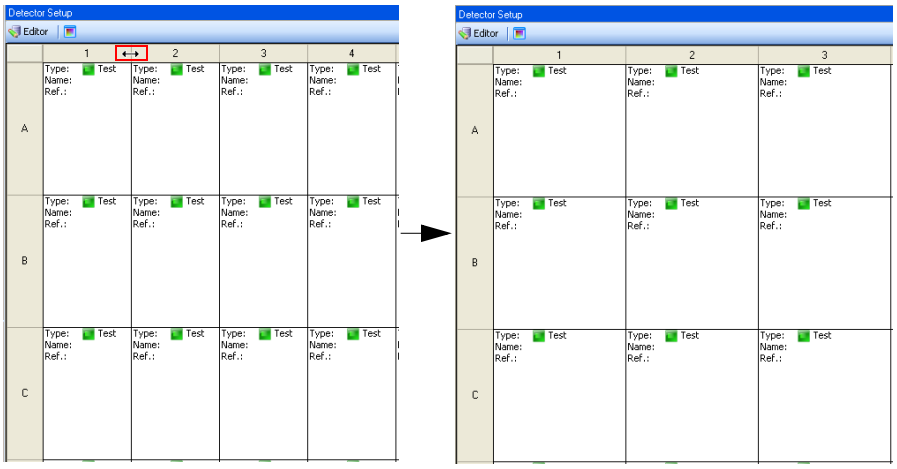
Test	Experiment reagents
Reference	A control or reference gene
NRC	No Reagent Control: negative control using only buffer, no primers/probes (detectors)

To Set Up the Detector:

1. Click **Detector Setup**.
2. Click **New**.

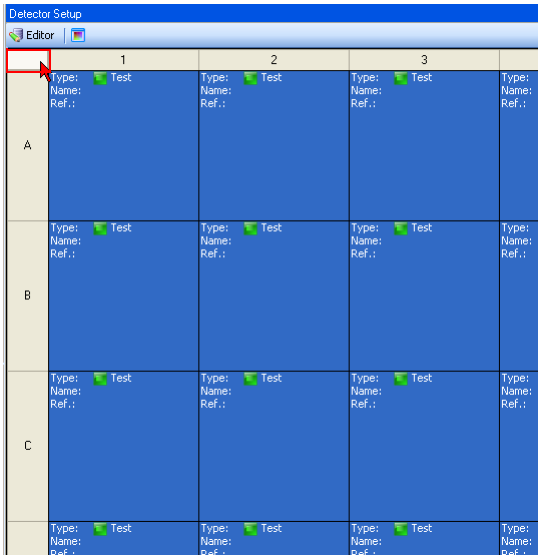


3. Choose the appropriate Container type and Container format.
4. Click **OK** to open the **Detector Plate** screen. OPTIONAL: Double-click between columns to expand.

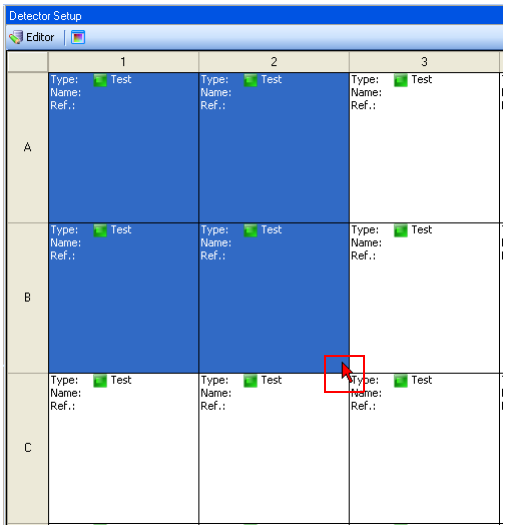


5 Select cells by performing one of the following:

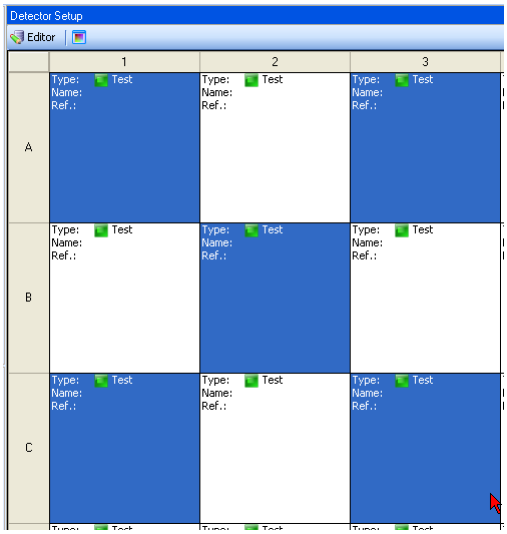
- Click and hold while dragging your cursor through cells.
- Click the upper left corner to select all the cells.
- Click individual cells while pressing the **CTRL** key.



Click the upper left corner to select all cells



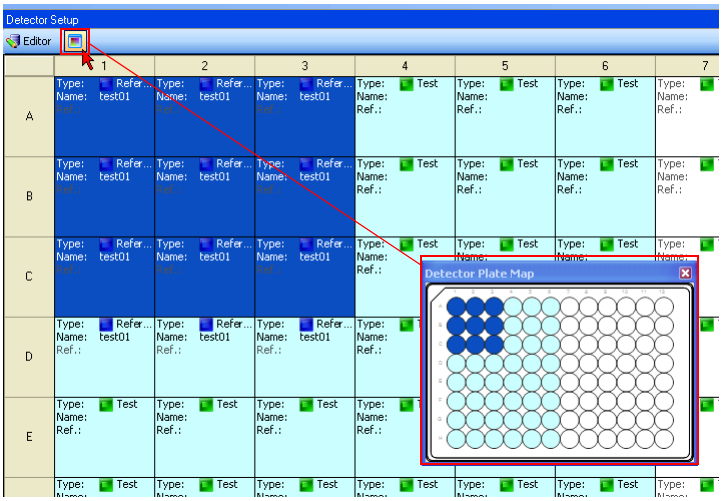
Click and drag to select



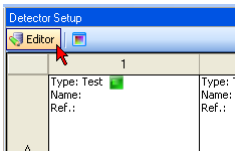
Press and hold the **CTRL** key while clicking individual cells

6 OPTIONAL: Click the **Detector Plate Map** icon .

The map opens and shows selected cell(s) relative to the entire detector plate.



7 Click **Editor**.



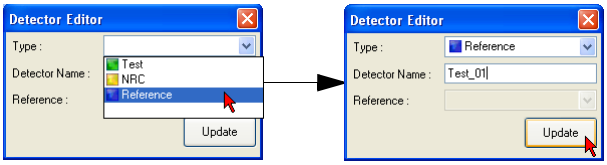
The **Detector Editor** opens.

8 Complete the **Detector Editor**:

a Select the appropriate type.

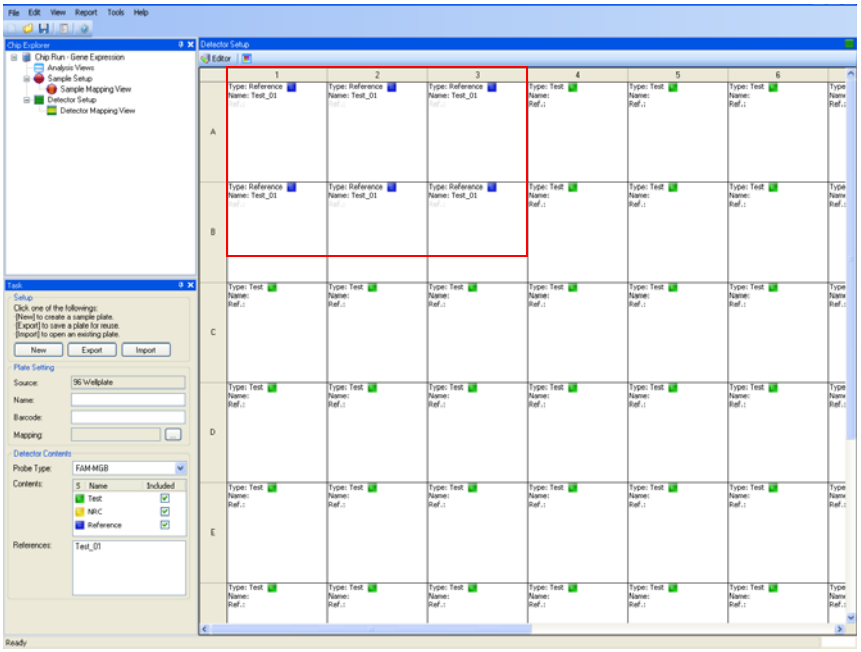
NOTE: If you want to identify a reference before moving on, see "Calculating Delta Ct Detector Values" on page 109.

b Enter the **Detector Name**.



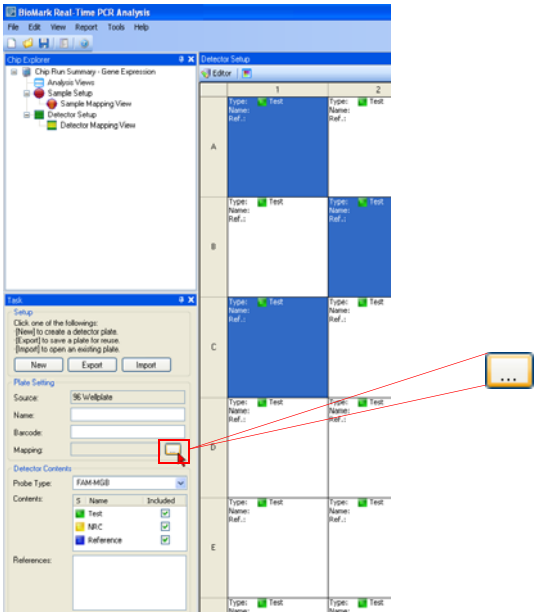
9 Click **Update**.

The **Detector Plate Setup** reflects the updates.



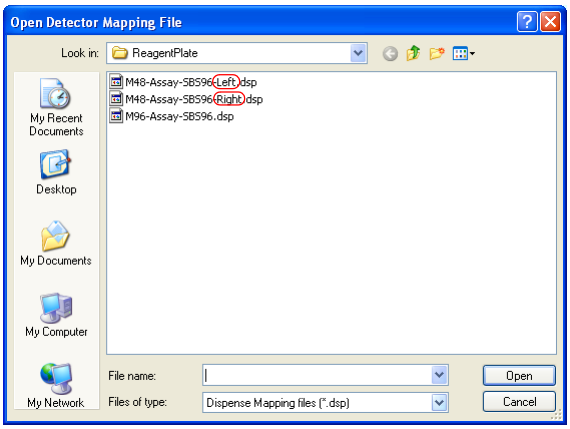
10 Close the **Detector Editor**.

11 Click the **Open Mapping File** icon.

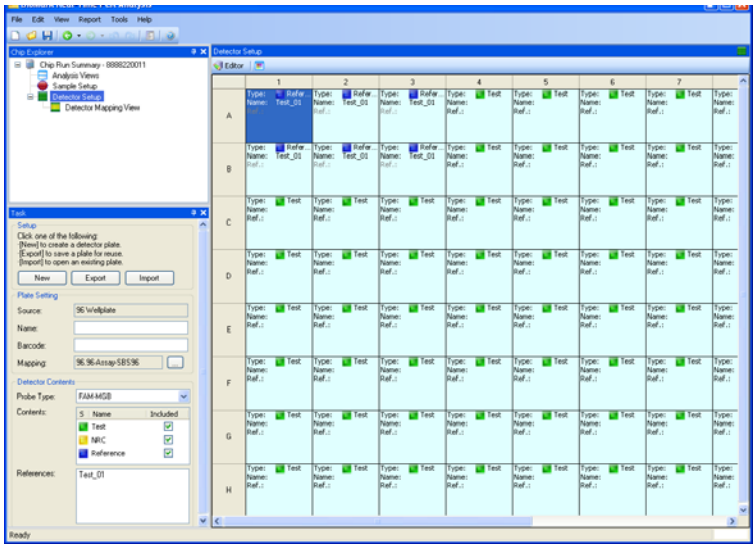


12 Double-click either left or right sample mapping file.

NOTE: If you are analyzing a 96.96 chip, select *M96-Assay-SBS96.dsp*.



Your selection is displayed in light blue (left or right) in the **Mapping Viewer**.



Your detector plate setup is complete.

NOTE: You can also copy and paste sample/assay names directly from Microsoft® Excel® spreadsheets.

Converting a Chip Run to a More Samples Run

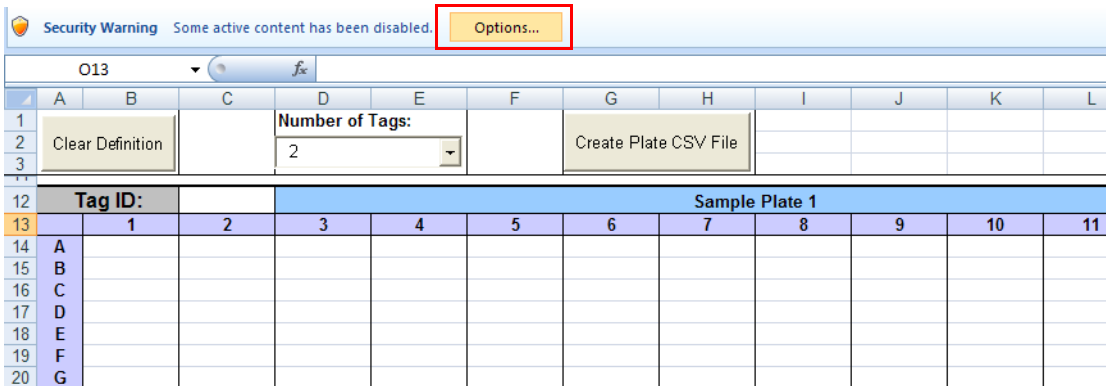
A **More Samples** run requires its own sample and assay setup, and Fluidigm provides the necessary Microsoft Excel setup templates for you.

Follow these steps to convert a chip run to a More Samples run:

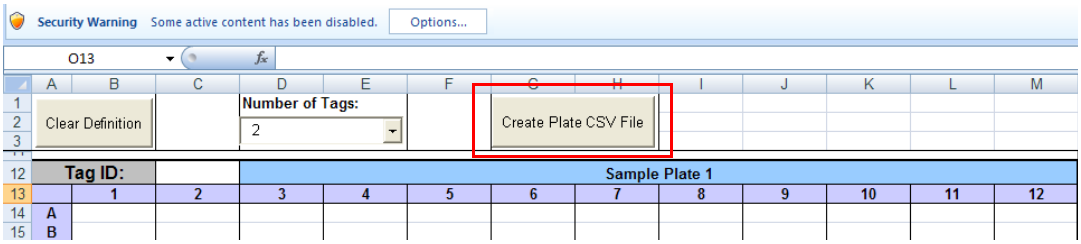
- Step 1: Set up your samples
- Step 2: Set up your assays
- Step 3: Import the samples and assays to the Analysis software

Step 1: Set Up Samples

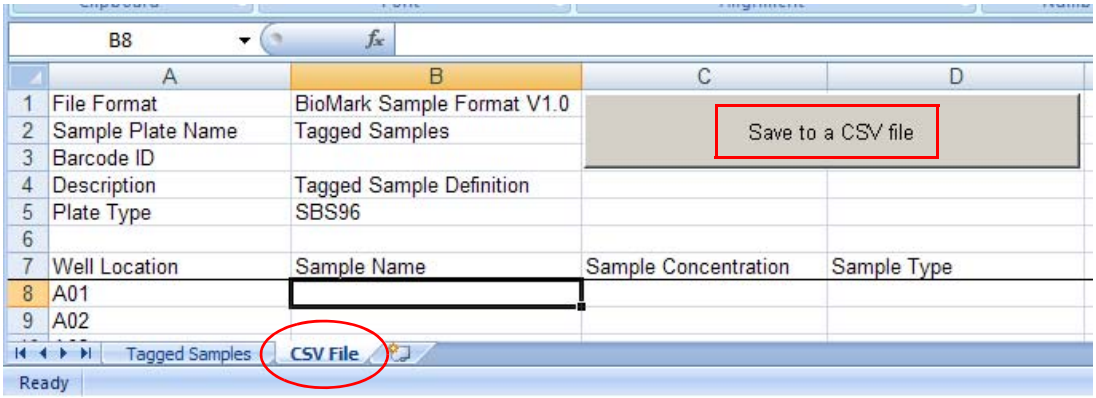
1. Go to *C:\Program Files\Fluidigm\BioMarkDataAnalysis\ApplicationData\FileFormats*.
2. Open **SamplePlateDefinitionForMoreS**.
3. Click **Options** to enable Active X (if prompted).



4. If prompted, select **Enable this content** in the Microsoft® Office Security Options dialog.
5. Click **OK**.
6. Edit the Microsoft Excel template to match your experiment.
7. Click **Create Plate CSV File**.



8 Open the new CSV file tab and double-check your annotations.



9 Click **Save to a CSV file** to save the file and select a location to save it.

Step 2: Set Up Assays

1 Open **AssayPlateDefinitionForMoreS**.
2 Edit the Microsoft Excel file to match your experiment.
3 Click **Create Plate CSV File**. A second **CSV file t**ab is added to the file.
4 Open the new CSV file tab and double-check your annotations.
5 Click **Save to a CSV file** to save the file and to select a convenient location for future retrieval.

Step 3: Import the Sample and Assay Templates

To Import the Sample template files:

1. From the Data Analysis software, open a chip run you want to annotate.
2. Select **Sample Setup**.
3. Click **Import** under **Task**.
4. Browse to the location where you saved your sample template.
5. Click **Open**.
6. Go to **File > Convert to More Samples Chip Run.**

To Import the Assay template files:

1. From the Data Analysis software, open a chip run you want to annotate.
2. Select **Assay Setup**.
3. Click **Import** under **Task**.
4. Browse to the location where you saved your assay template.
5. Click **Open**.
6. Go to **File > Convert to More Samples Chip Run**.

Viewing Chip Run Data in the Data Analysis Software

3

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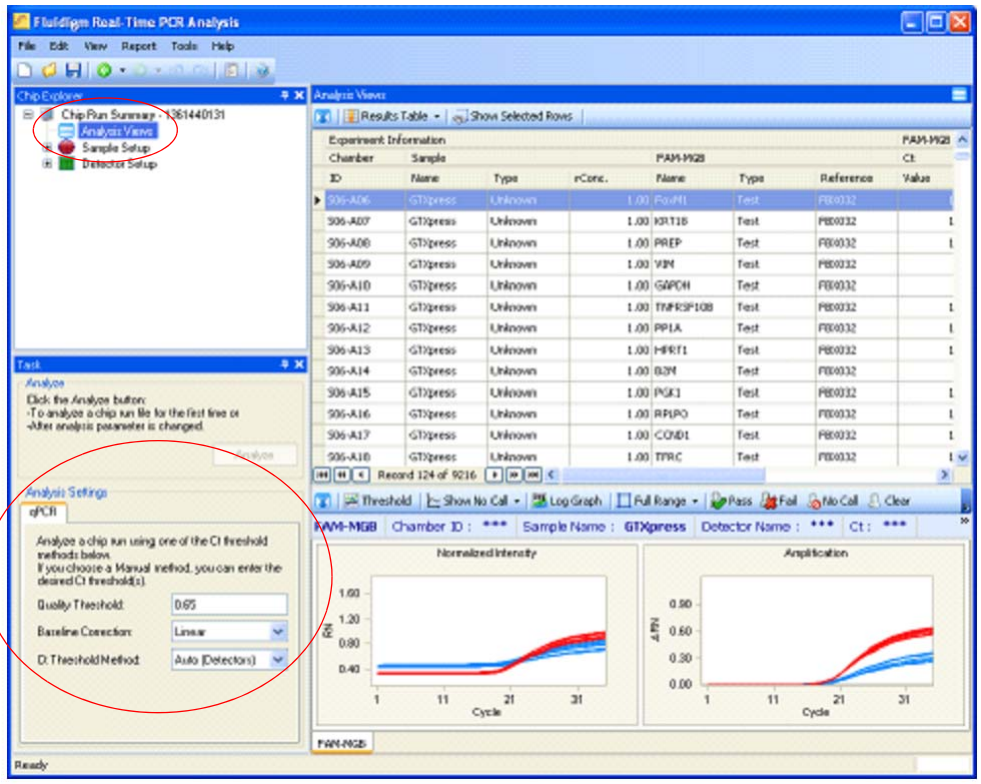
Working with Analysis Settings

You can customize these analysis settings:

- Quality Threshold
- Baseline Correction
- C_t Threshold Method

To change the settings:

1. Launch the Real-Time PCR Analysis software.
2. Click **Analysis Views**. Analysis settings are located under **Task**.



Changing the Quality Threshold

To change the quality threshold:

1. Click **Analysis Views**.
2. Under **Analysis Settings**, enter a different value in the **Quality Threshold** field.

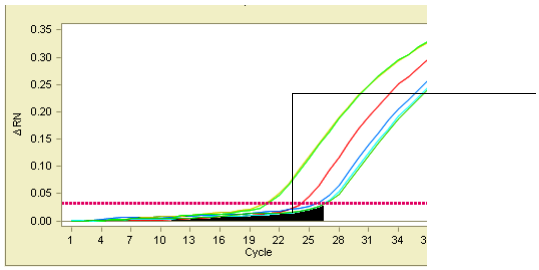
Changing the Baseline Correction

To change the baseline correction:

1. Click **Analysis Views**.
2. Under **Analysis Settings**, select a different option in the **Baseline Correction** field.

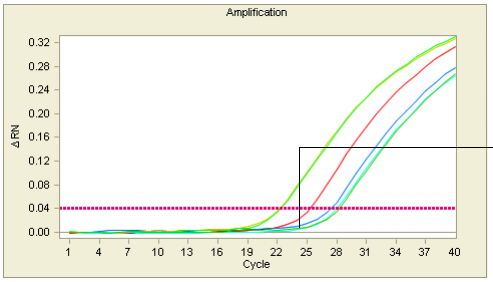
Linear [default]	Produces higher C_t values when the amplification is low. Linear baseline correction eliminates baseline "drift" by flattening the baseline.
Linear (Derivative)	An additional method of baseline correction with a more robust handling of nonlinear baselines and their impact on C_t estimates.

The difference between **Constant** and **Linear** baseline corrections is shown here:



Constant baseline correction

Baselines are rising in the **Constant** correction (shown as black fill).



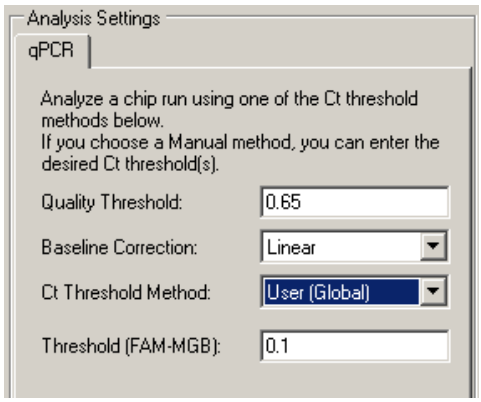
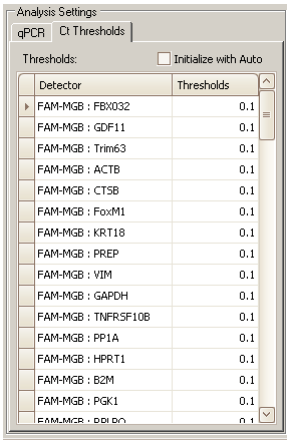
Linear baseline correction

Baselines are flattened in the **Linear** correction

Changing the C_t Threshold Method

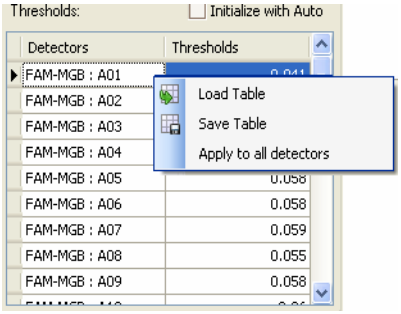
To change the quality threshold:

1. Click **Analysis Views**.
2. Under **Analysis Settings**, select a different option in the **C_t Threshold Method** field.

Auto Global	Automatically calculates a threshold that is applied to the entire chip.
Auto Detectors	Independently calculates a threshold for each detector on a chip. You must enter a unique detector name in the **Detector Editor** during detector setup.
User Global	Allows you to manually adjust the threshold when searching for the C_t rise in slope. The value is applied to all the detectors. 
User Detectors	Allows for tighter control when searching for the C_t curve's rise in the slope. You can individually set the threshold for each detector on the **Ct Thresholds** tab. 

Saving or Loading Threshold Settings

To save your detector threshold settings for use with another chip run, right-click anywhere in the threshold table and select **Save Table**. Name the file and choose the location where you want to save the file.



Right-click anywhere in table to open menu

To load your saved detector threshold settings, select **Load Table**. Browse to the location of the settings file you want.

Working with Analysis Views

There are three different views:

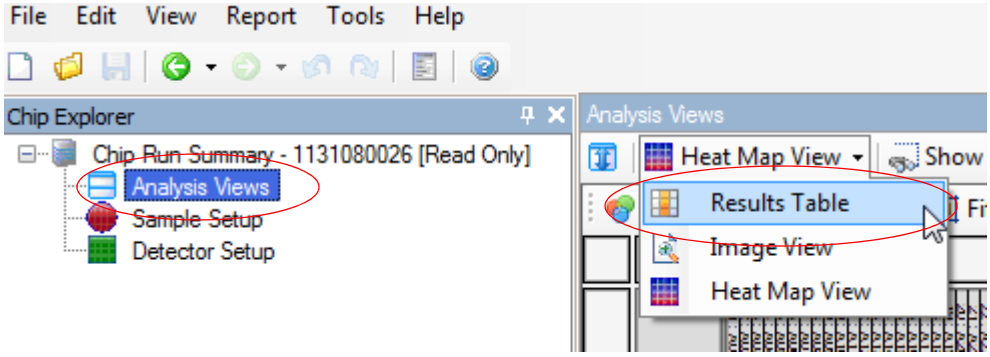
- **Results Table** -- view results in one table
- **Image View** -- view images from individual cycles
- **Heat Map View** -- view color-coded C_t values

Click the **Expand/Collapse** button to display any view at full size or as split screen.

Using the Results Table

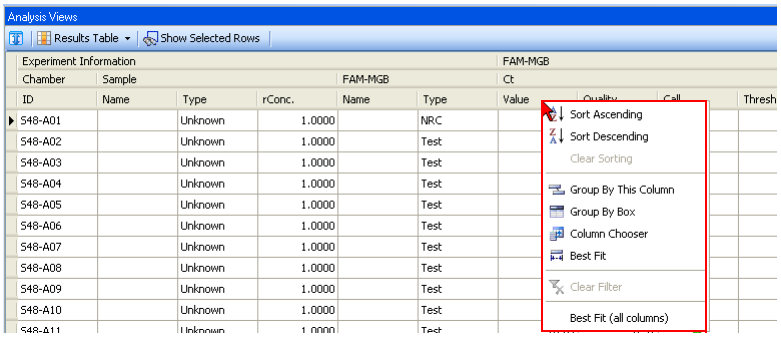
To access the Results Table:

1. Click **Analysis Views**.
2. Select **Results Table**, if it is on a different view.



In the **Results Table** view, right-click a column header to:

- Adjust columns, see page 58
- Group columns, see page 59
- Sort columns, see page 65
- Column Chooser, see page 66
- Customize search filters, see page 68



In this example, right-click the Value column header to open the options menu.

Resizing Columns

Using the Cursor

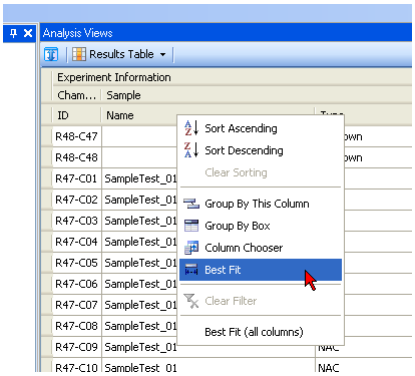
- Position the cursor on a column edge. When the cursor changes to a double arrow, hold and drag the column bigger or smaller.

Or

- Double-click the column edge to adjust the column to precisely fit the contents.

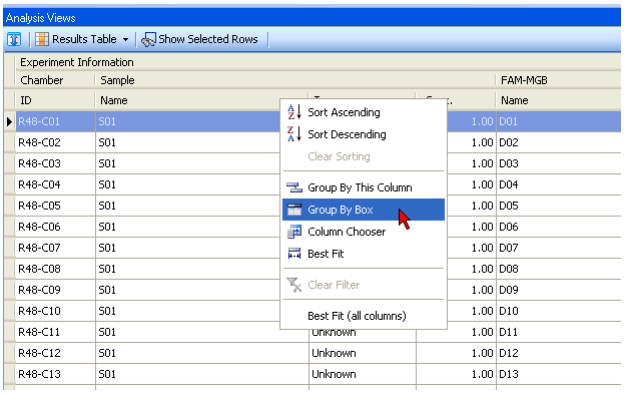
Using "Best Fit"

- One column: right-click a column header and select **Best Fit**. The column automatically adjusts to precisely fit the contents.
- All columns: right-click a column header and select **Best Fit (all columns)** to adjust all columns to precisely fit the contents.

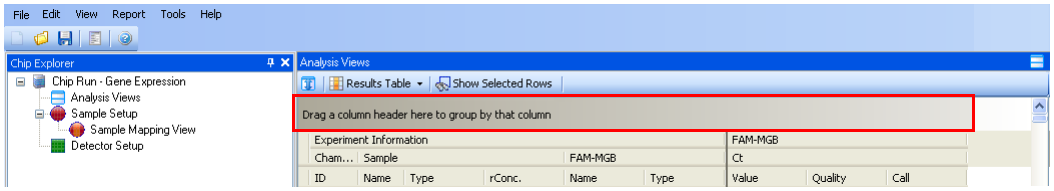


Grouping Two or More Columns

1. Right-click on any column header.
2. Click **Group by Box**.

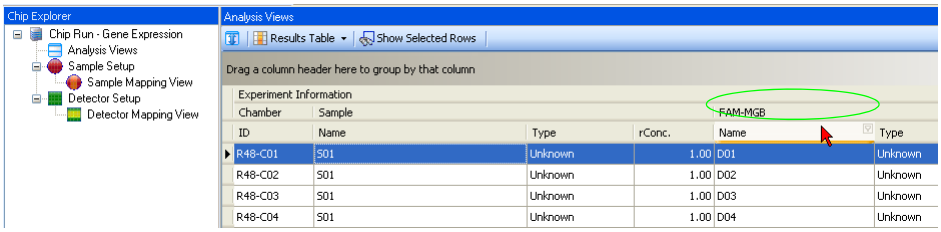


The grouping bar appears.

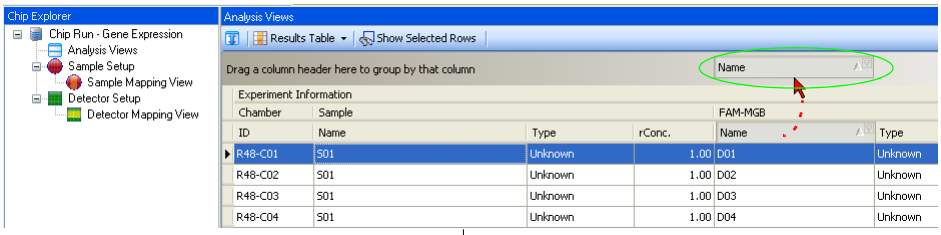


3 Click the column header that you want to group and, while holding down the mouse button, drag it to the bar as shown below.

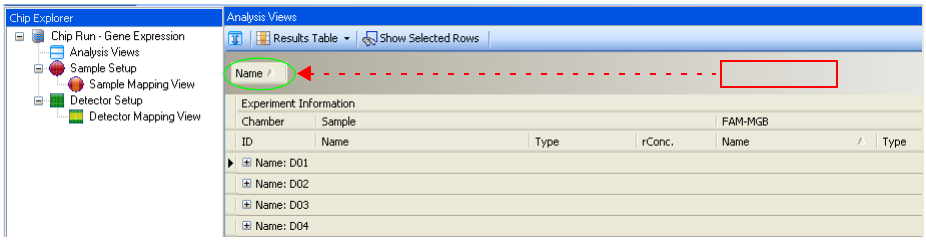
a. Click and hold mouse button on header



b. Drag to any place on the bar

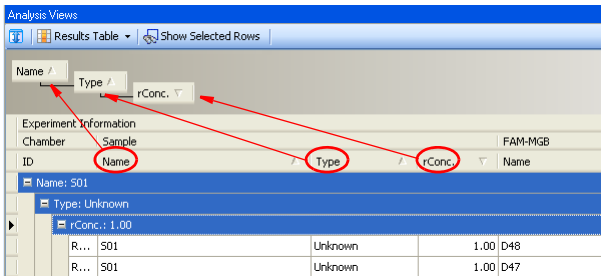


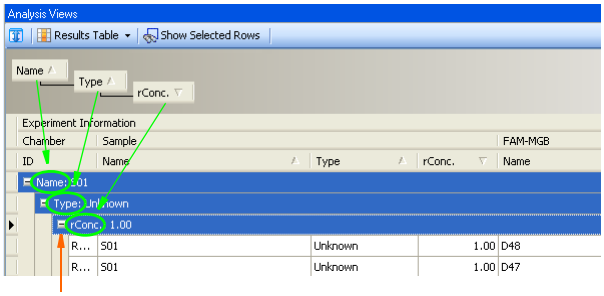
c. Release mouse button and header snaps to position at the left



The data are now grouped by name in the **Results Table**.

4 Group as many elements as you like by dragging and dropping, as in the example below.

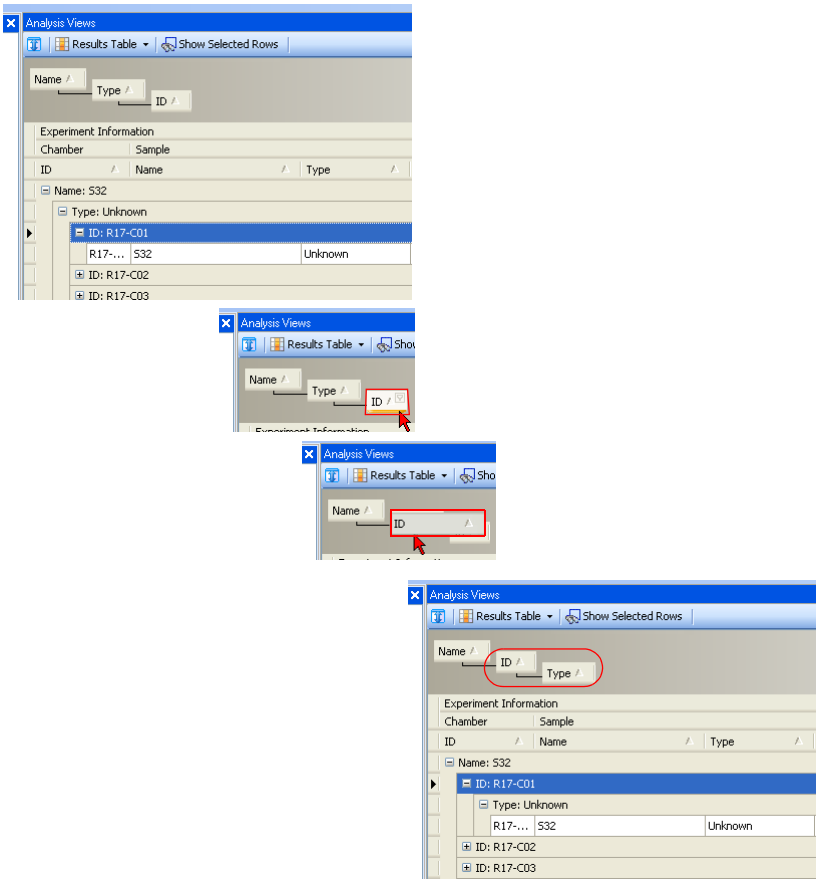




Click + (plus) or – (minus) to expand/collapse the windows.

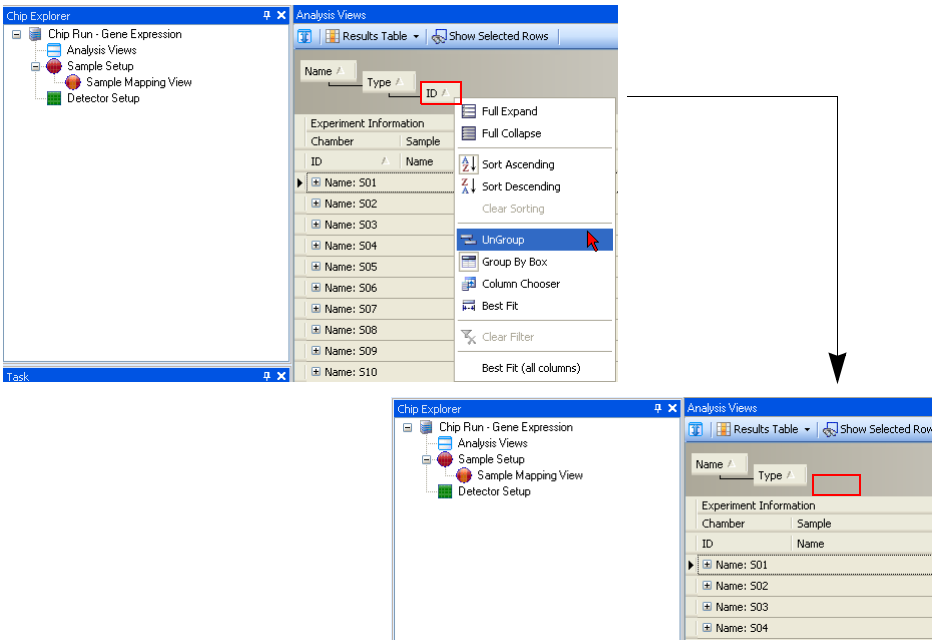
5 Drag and drop one header element over another as shown below to change places (hierarchy). The hierarchy dictates how the data displays as you expand windows.

In this example, the **ID** header is dragged over the **Type** header and then dropped. They exchange places as a result.



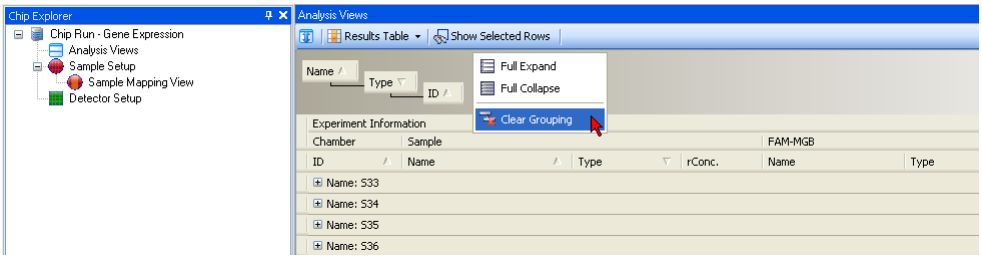
Ungrouping One Header

1. Right-click a header within a group.
2. Click **Ungroup** to remove the header from the group.



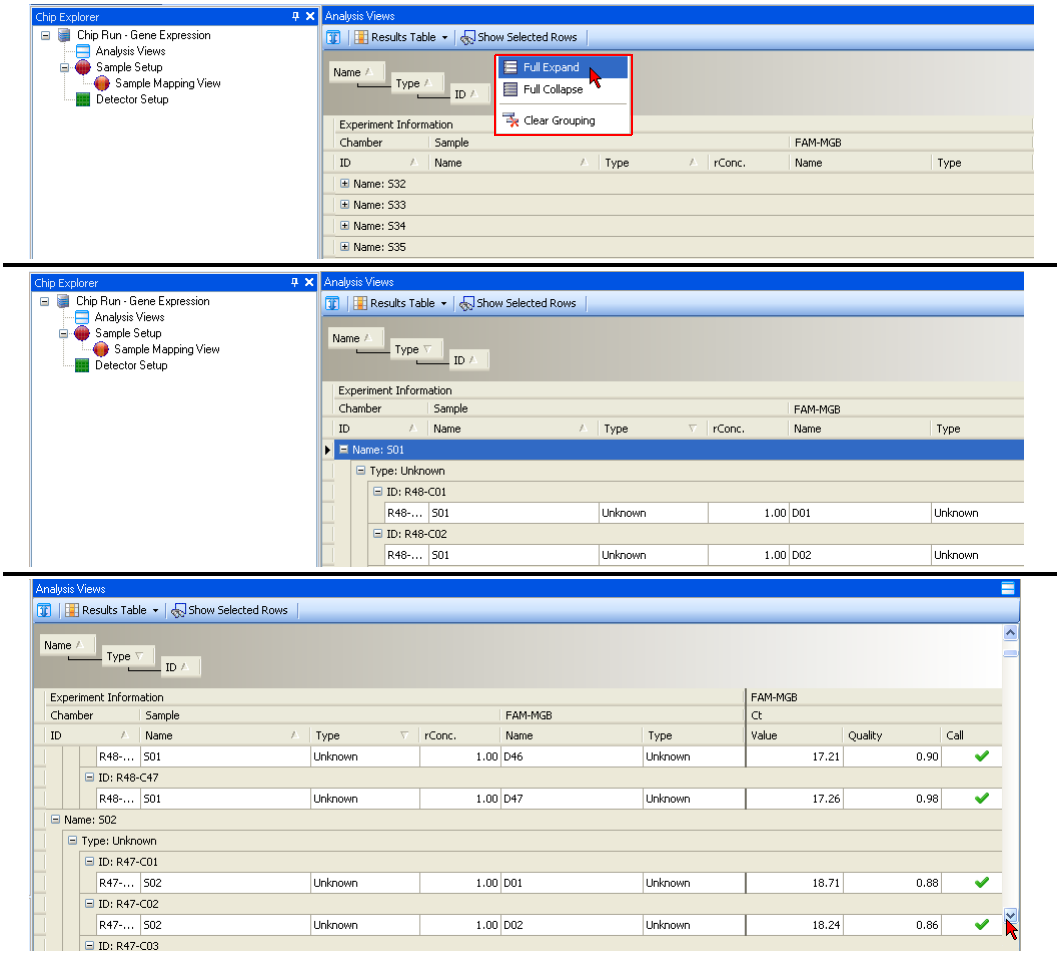
Ungrouping All Headers

1. Right-click anywhere on the grouping bar.
2. Click **Clear Grouping**.



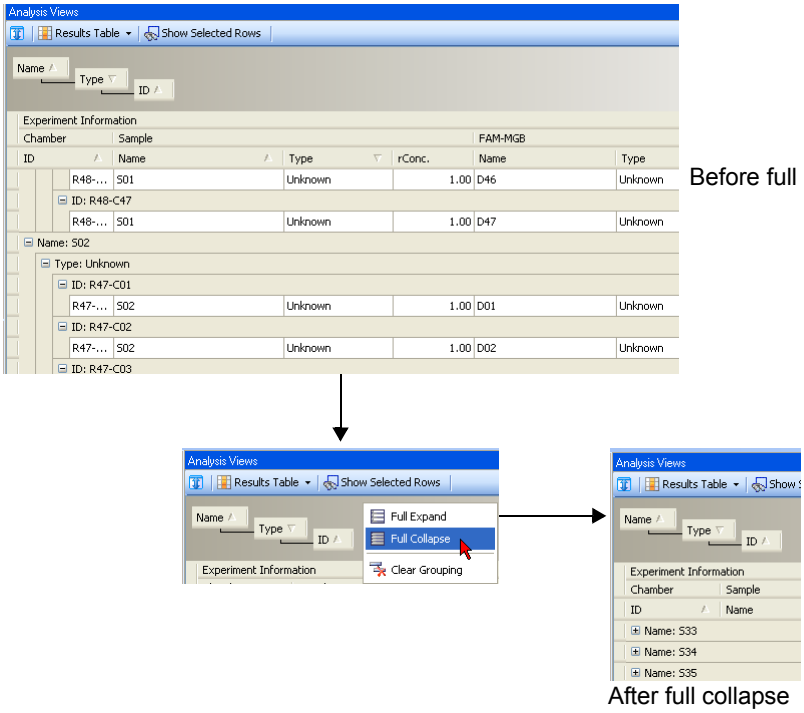
Expanding and Collapsing All

1. Right-click anywhere on the grouping bar.
2. Click **Full Expand**. The grouped windows expand as shown below.



3. Collapse all by right-clicking anywhere on the grouping bar.

4 Select **Full Collapse**.



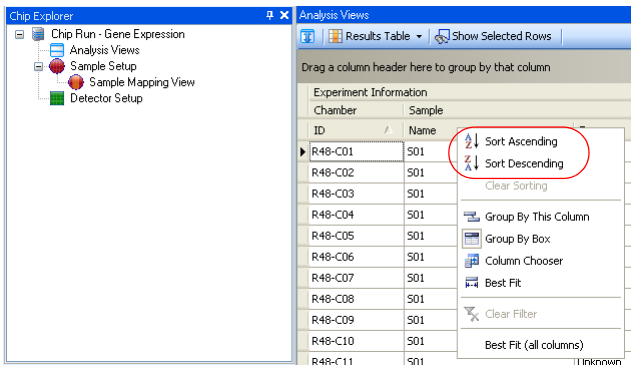
Before full collapse

After full collapse

Sorting Columns

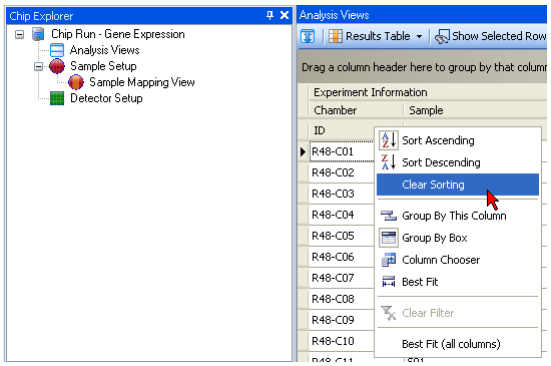
1 Right-click a column header.

2 Choose either **Ascending** or **Descending** to sort that column accordingly.



Unsorting Columns

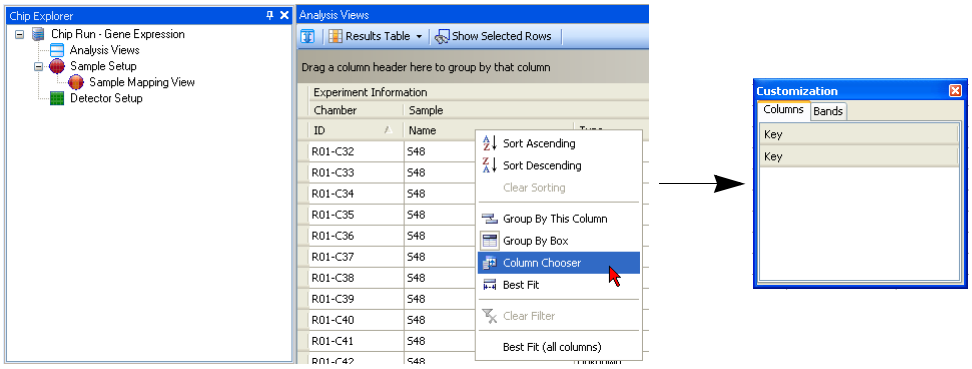
1. Right-click a sorted column header.
2. Click **Clear Sorting**.



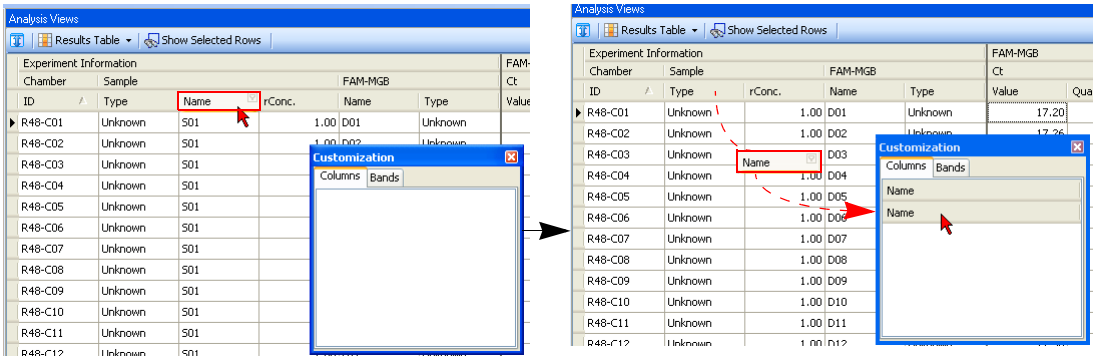
Column Chooser

Depending on how you set up your sample plate and detector plate, you can have 20+ columns in the **Results Table**, all of which are not viewable at once. To temporarily remove columns not of immediate interest, follow the procedure below.

1. Right-click a header.
2. Click **Column Chooser**. The **Customization** dialog opens.



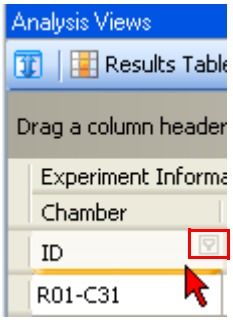
3. Drag and drop unwanted column headers onto the **Customization** dialog.



4 Replace the column headers by dragging them from the **Customization** dialog to their original position.

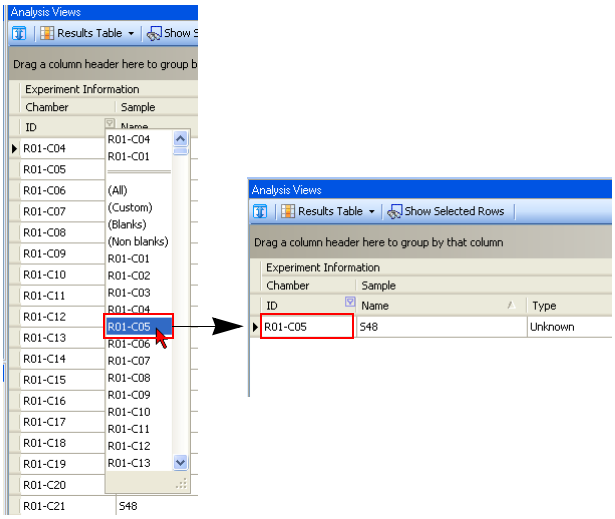
Dropdown Menus on the Column Headers

Each column header has a dropdown menu. Place your cursor over a header to reveal the symbol.

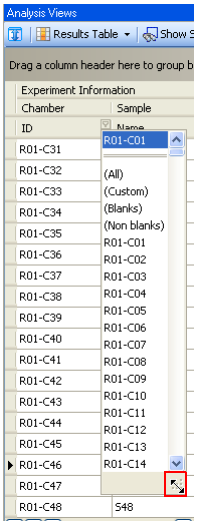


1 Click the dropdown menu symbol to display the menu.

2 Click a location to go to that location.



3 Click and drag the menu to size it.

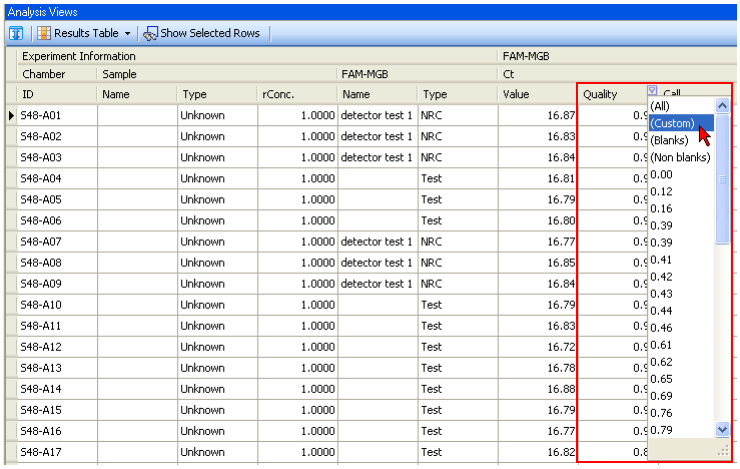


Custom Filters

Use filters to narrow your search for a particular parameter. In the following example, we isolate C_t quality values below 0.8.

1 Click the **Quality** header menu.

2 Click **Custom**.



The Custom AutoFilter dialog box opens.

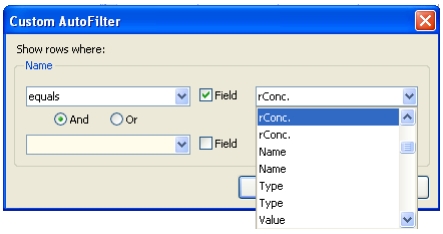
3 Delimit your search:

a Select a filter.

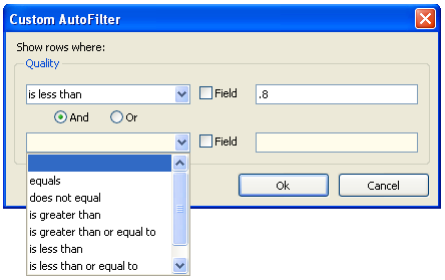
b Enter the target value (0.8 in this example).

Or

c Click the **Field** checkbox to activate the menu and select a filter.



4 *Optional*: Continue delimiting your search by clicking **And/Or** and then selecting filters from the menus.



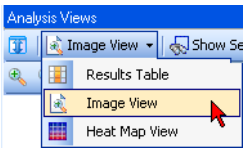
5 Click **OK**.

Using the Image View

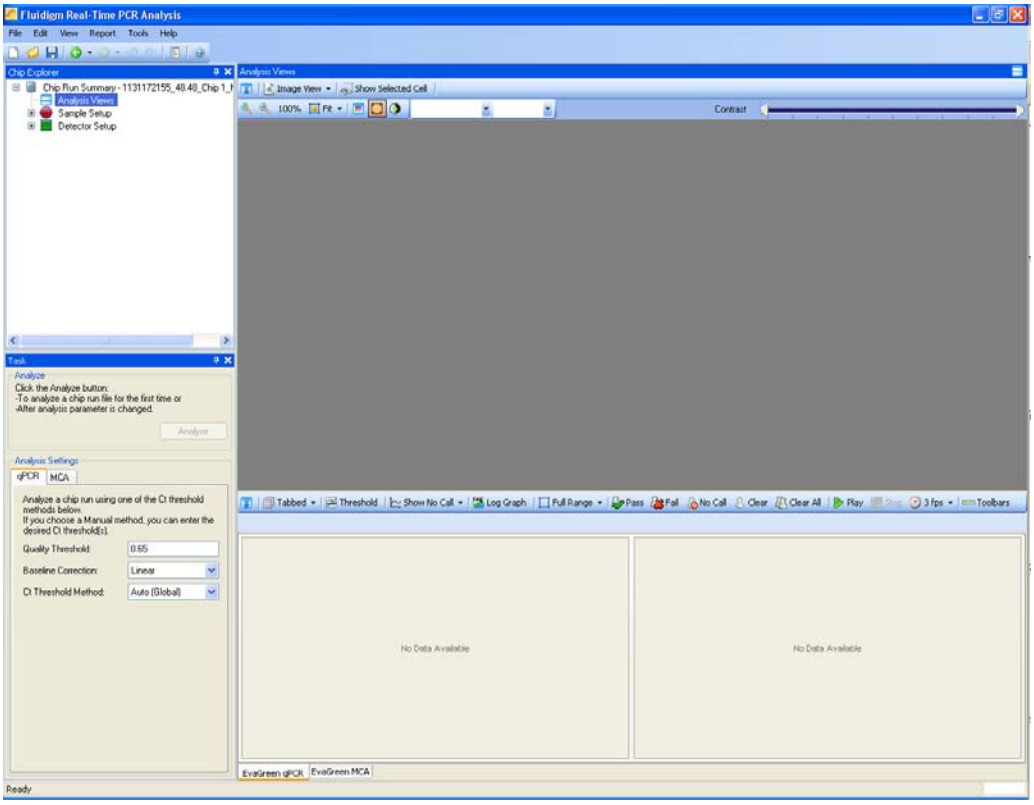
View images from individual cycles in this window.

1 Click the **Results Table** menu.

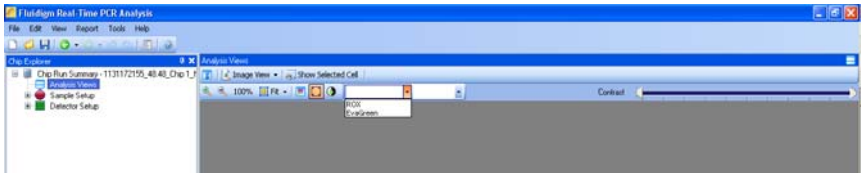
2 Click **Image View**.



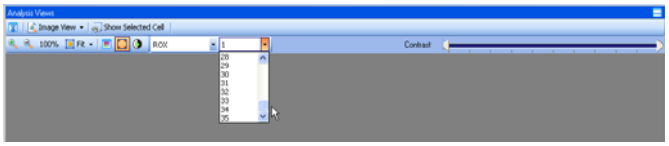
The default **Image View** opens.



3 Select a dye.

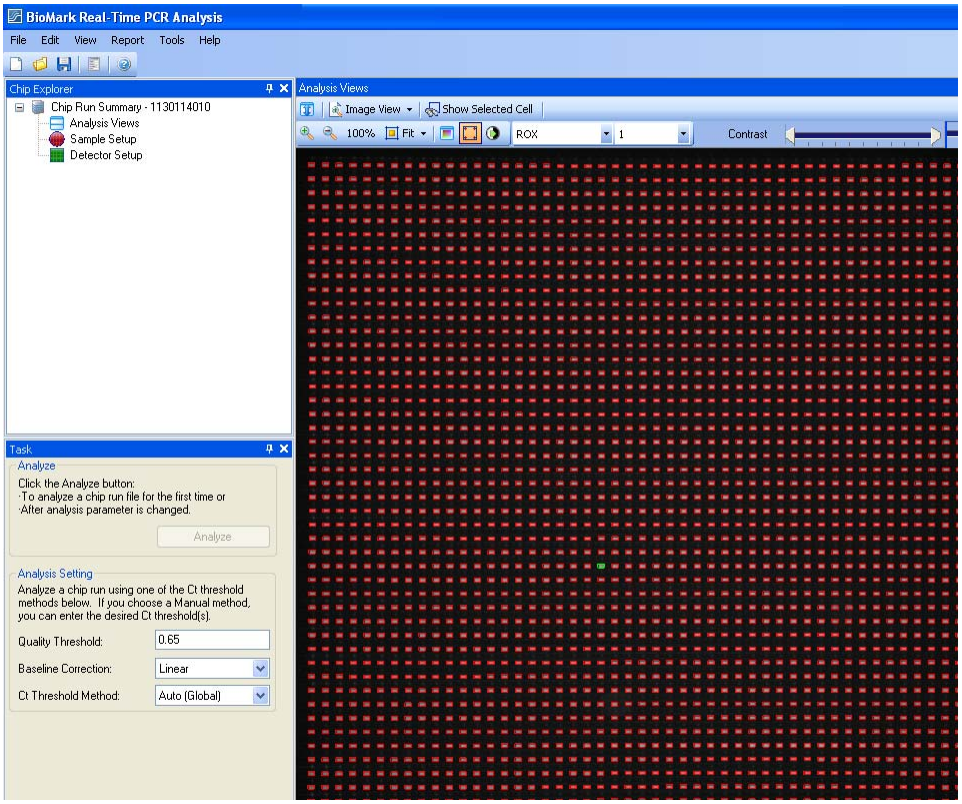


4 Select a cycle number from the **Cycle Selection** menu.



IMPORTANT: An image displays only after you have selected a dye and a cycle number.

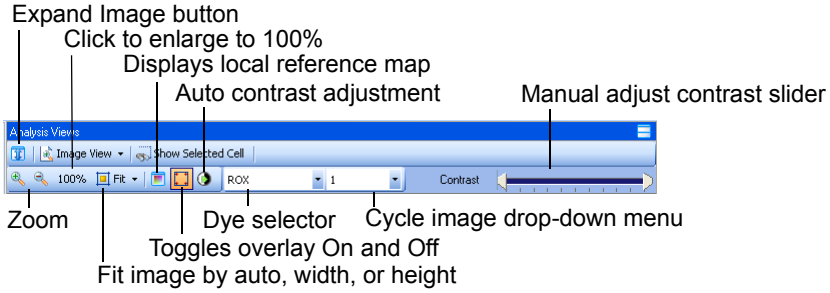
A representation of the chip displays in the Image View.



5 *Optional*: Click the double arrow to expand the image.

Image View Tool Bar

Elements of the Image View tool bar are shown below.

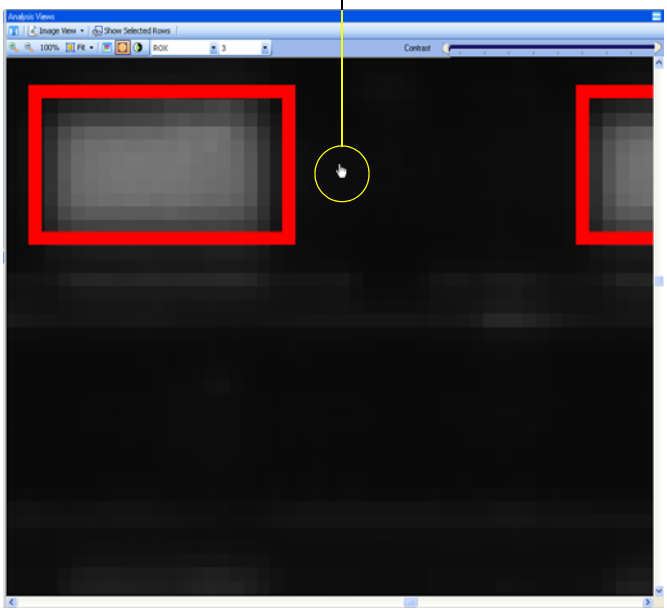


Zoom

You can increase or decrease the image view size in several ways:

- Multi-clicking the magnifying glass buttons (+ and –).
- Click the 100% button.
- Clicking the Fit button to fit image to width.
- Clicking inside Image View and then rolling the mouse scroll wheel (up/backward = larger, down/forward = smaller).

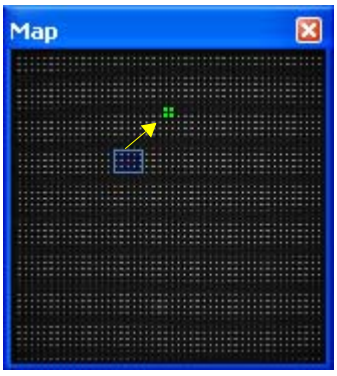
Click and hold anywhere on the image. When the hand icon appears, drag the image until it is centered on the cell of interest.



Location Reference Map

Use the location map to reference your cell of interest within the entire framework of the chip.

1. Click the Location Reference map icon to open the map.
2. Click and drag the blue rectangle to a location of interest. In the example below, the blue rectangle within the Location Reference map is dragged to the green cells which enlarges the green cells in the Image Viewer.



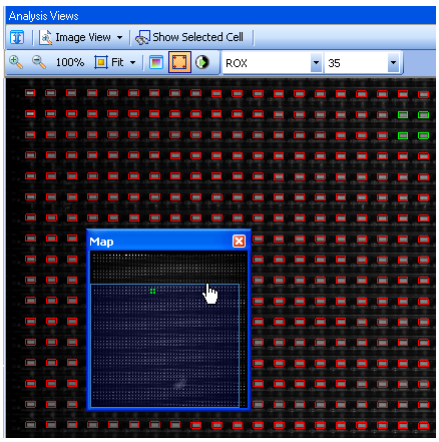
Drag blue rectangle to area of interest

Dragging the blue rectangle to an area of interest enlarges that area in the Image Viewer as shown here.

Adjusting the Size of the Location Reference Map

The size of the image in the Image Viewer determines the size of the blue rectangle in the Location Reference Map.

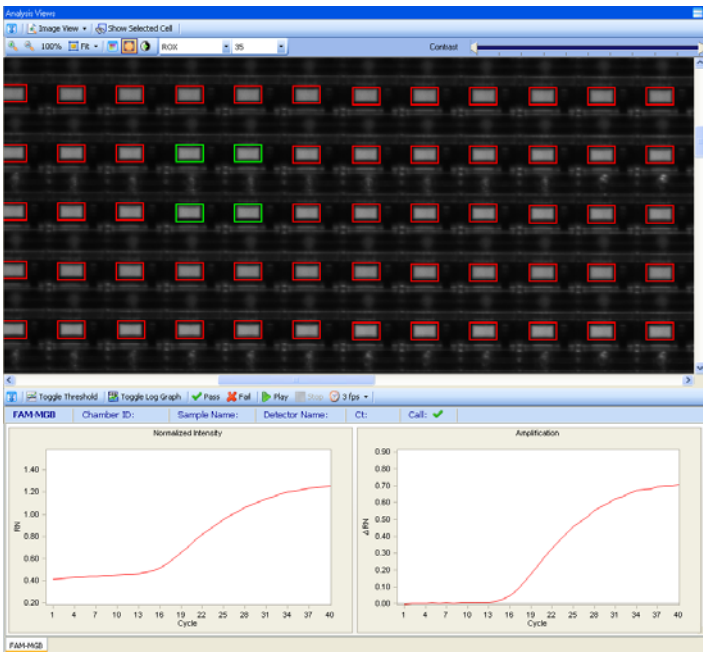
In the example below left, the image is has not been zoomed so the blue rectangle on the map is large. In the example below right, the image has been enlarged (by clicking in the Image View and then rolling the scroll wheel on the mouse).



Here, the blue rectangle is large and cannot be dragged with much accuracy.

Here, the image has been enlarged so that the blue rectangle is smaller and easily dragged to an area of interest.

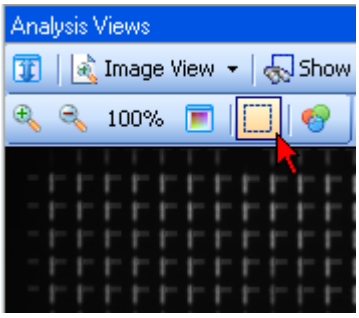
NOTE: Selected cells in the Image View are also displayed in the Graph View as shown below.


Enlarged image in the Image View, with four cells selected.
Graph View displaying the amplification plots and dye intensities of the four selected cells.

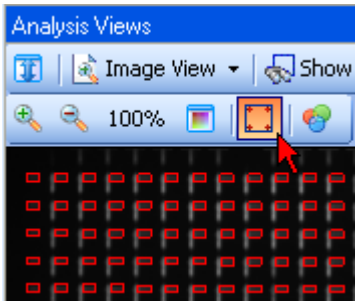
Raw data, amplification plots, and dye intensities are displayed in the Graph View when cells are selected in the Image View.

Overlay

1. Click the Overlay icon to activate the red-square grid.
2. Click the Overlay icon again to inactivate the red-square grid.



Toggle grid *off*



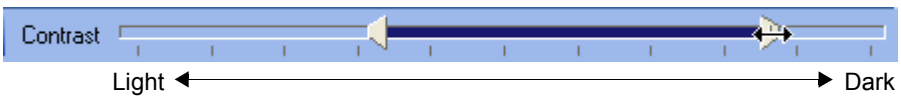
Toggle grid *on*

Contrast

Adjust image contrast:

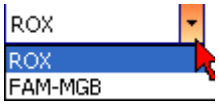
- Click the Auto-Contrast icon.

  Or,
- Move the contrast sliders by placing your cursor over a slider, then click and drag.



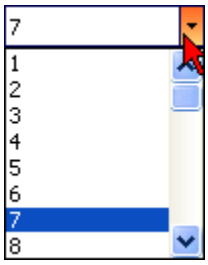
Dyes

Change the dyes.



View Image in Each Cycle

Use the menu to select an image to view. Select number 7 in the menu, for example, and the image taken at cycle 7 displays in the **Image Viewer**.

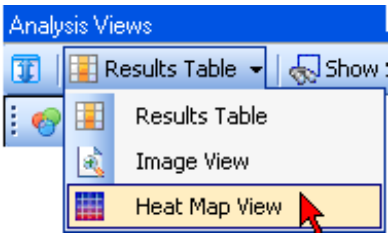


Using the Heat Map

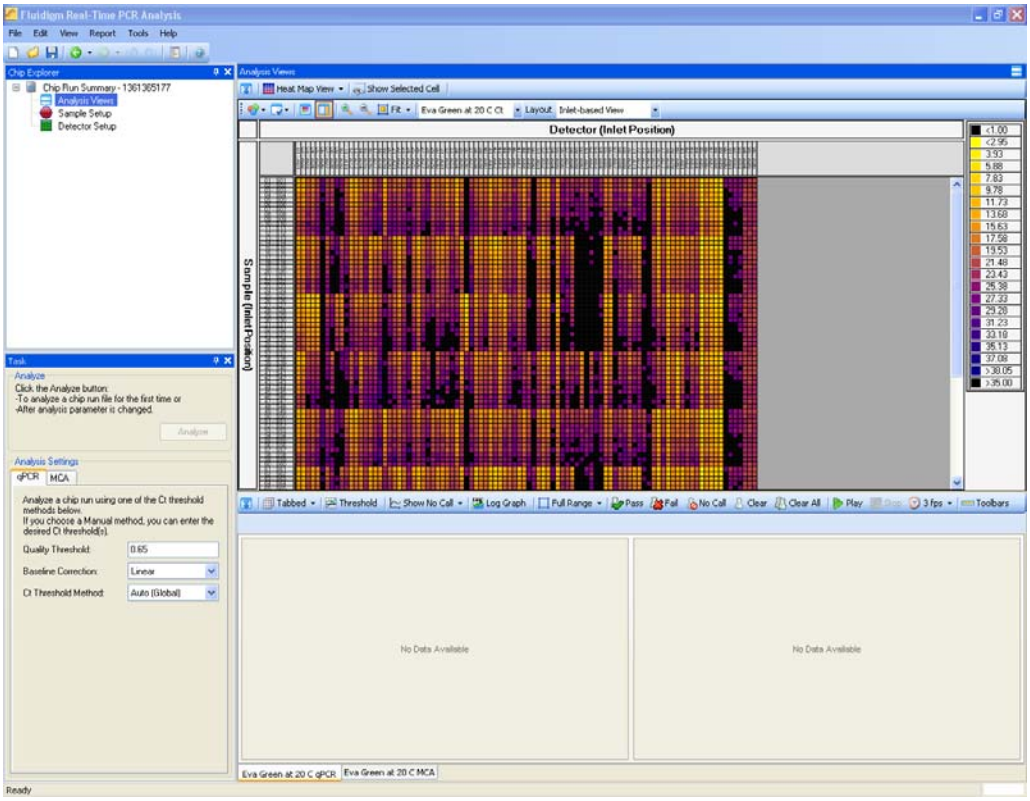
The heat map color codes C_t values for easy reference.

To access the heat map view:

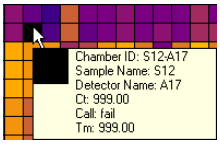
1. Click **Analysis View** in the Real-Time PCR Analysis software.
2. Go to **Heat Map View**.



The default heat map opens.



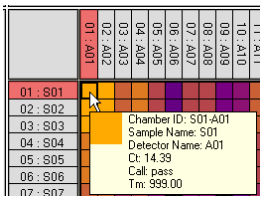
NOTE: A black square indicates no C_t value or a value outside of the spectrum range, as shown in the example below. Also, negative controls that do not show amplification appear as black squares.



NOTE: An **X** signifies an amplification curve marked as fail.

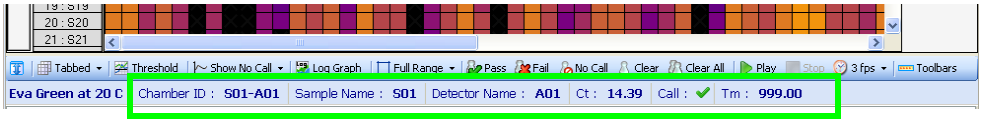
3 *Optional:* Click the double arrow to expand the image.

4 *Optional*: Hold your cursor over a cell of interest and an information dialog box opens; click the cell and the information appears on the task bar.

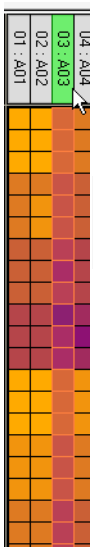


Hold the cursor over a cell to open the information dialog box...

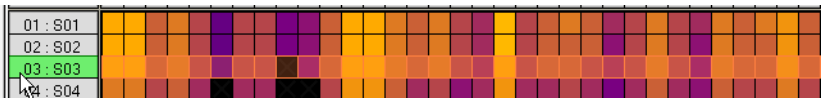
...click the cell to display the information on the bar also.



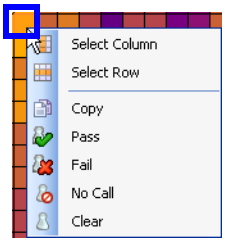
5 *Optional*: select a row or a column by clicking an inlet or using the right-click menu as shown below.



Click to select a column

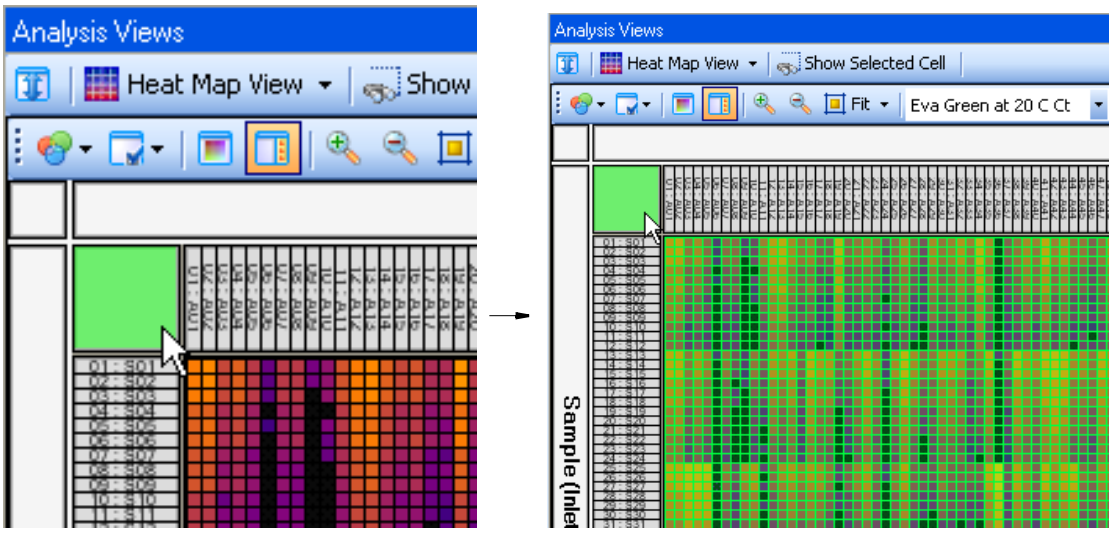


Click to select a row

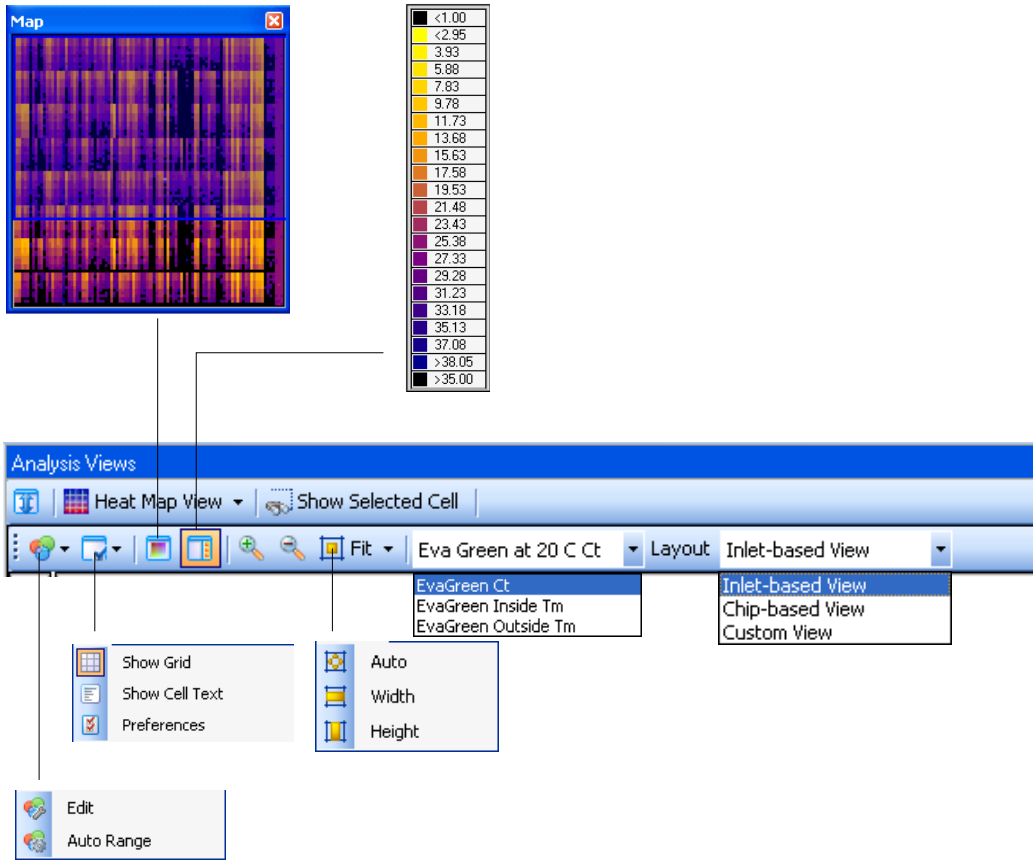


Hover cursor over cell and right-click to open options menu.

6 *Optional:* Click the upper left corner in the heat map to select all cells.



Heat Map View Tool Bar



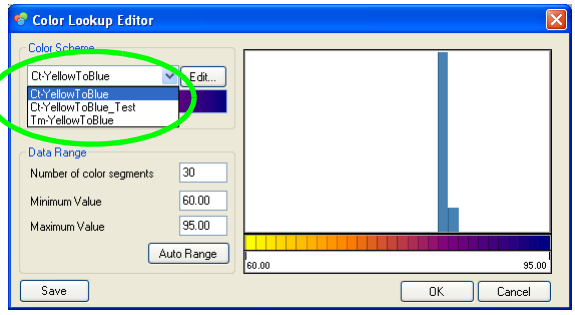
Color Lookup Editor

You can define a range of valid C_t or T_m values using the **Color Lookup Editor**.

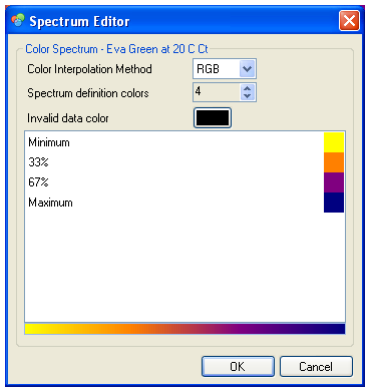
1 Click the **Color Lookup Editor** button .

2 Choose **Ct-YellowToBlue** or **Tm-YellowToBlue**.

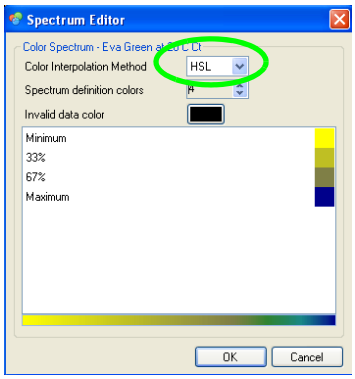
3 Click **Edit**.



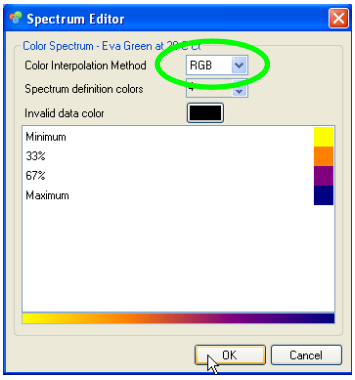
The **Spectrum Editor** opens.



4 Choose **RGB** (red, green, blue) or **HSL** (hue, saturation, lightness).



HSL



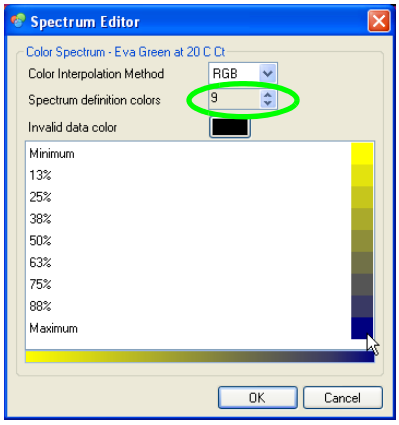
RGB

5 *Optional:* Change the percentage increments between colors by changing the number.

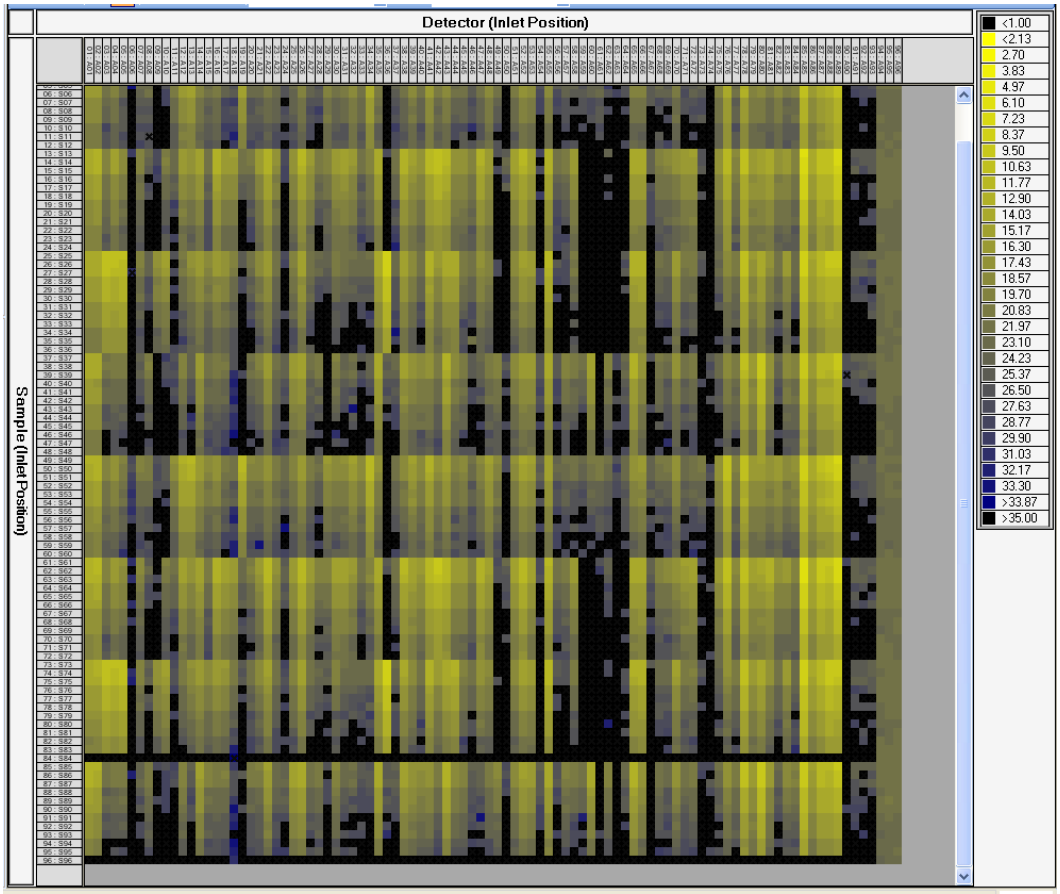
a Click **Edit**.

b Change the value (from 1 to 20).

c Click **OK**.



d Click **OK**.



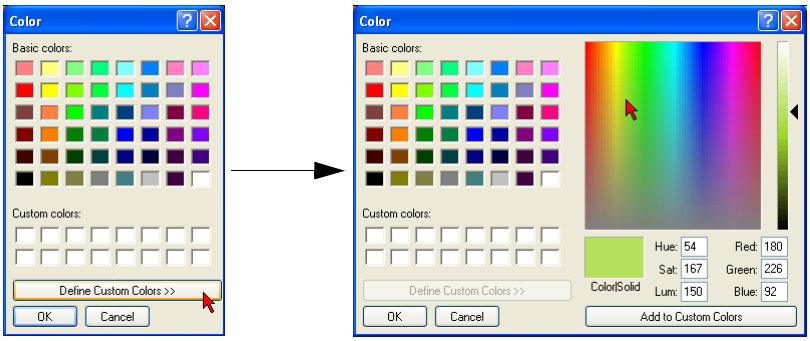
The change is reflected in the heat map and in the legend.

6 *Optional:* Click **Invalid Color Data** to change the color of failed cells.

a Click a color square.

Or

b Click **Define Custom Color** to pick a color other than a basic color.

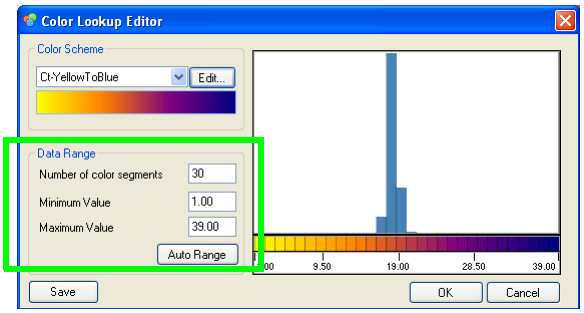


c Click **OK**.

Color Range Pane in the Color Lookup Editor

You can change the following parameters:

- Number of color segments
- Minimum value
- Maximum value
- Auto Range



Changing the Number of Color Segments

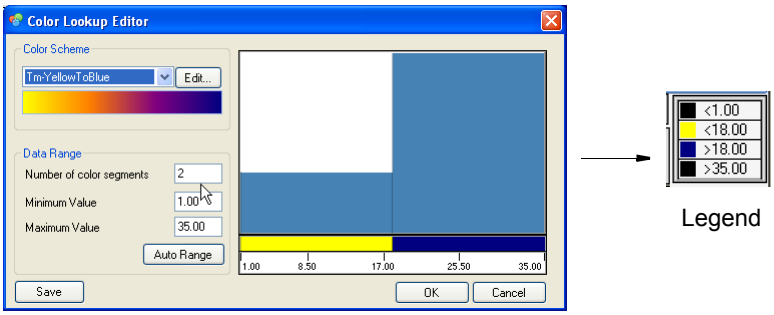
Change the segments shown in the heat map.

1 Type a value (2 minimum).

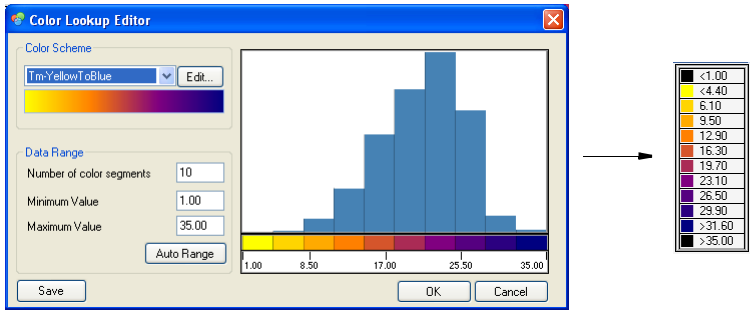
2 Click **OK** to reflect changes in the legend.

The examples below illustrate that the greater the number of color segments, the finer distinction between legend values.

1 Color Segment



10 Color Segments

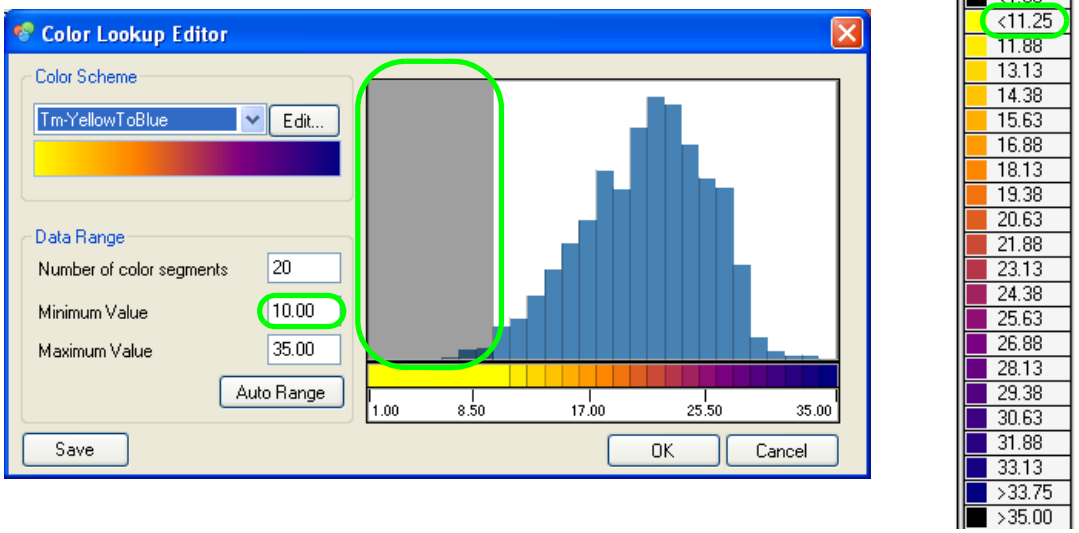


Changing Minimum Values

Change the minimum value when you want to exclude a segment from the lower range. For example, changing the value from 1 to 10, excludes any C_t value from 1 to 10 as the example below illustrates.

1. Enter a value.
2. Click **OK**.

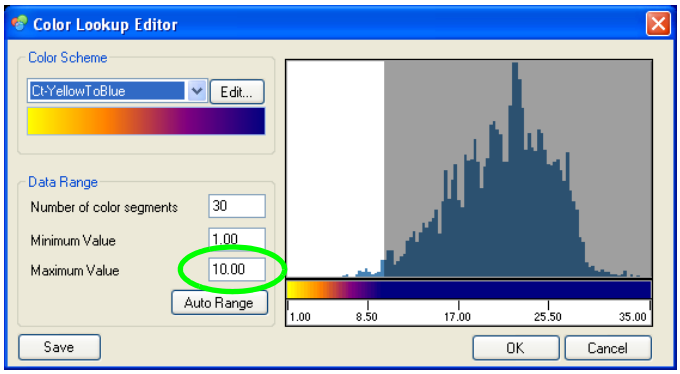
Segments with values from 1 to 10 have been eliminated as the minimum acceptable value is now greater than 10. As a result, the lowest value on the legend is now over 10.



Changing Maximum Values

Change the maximum value when you want to exclude a segment from the higher range (from 1 to 39). For example, changing the maximum value from 35 (default value) to 10, any C_t or T_m values above 10 are excluded (gray area), as the example below illustrates.

1. Enter a value.
2. Click **OK**.

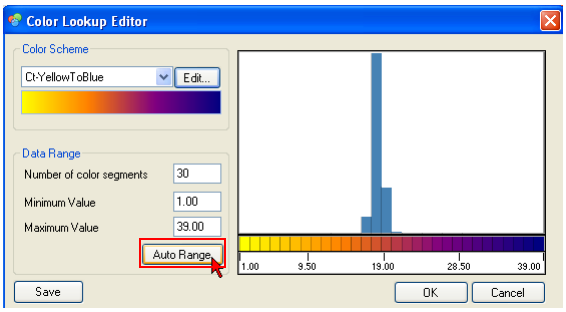


When you click **OK**, the heat map and the legend reflect the change also.

Using Auto Range

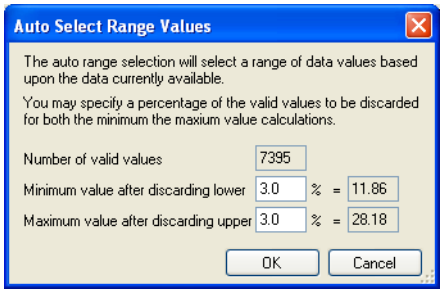
Auto range allows you to eliminate a percentage of the upper and lower ranges of all valid C_t or T_m values.

1 Click **Auto Range**.

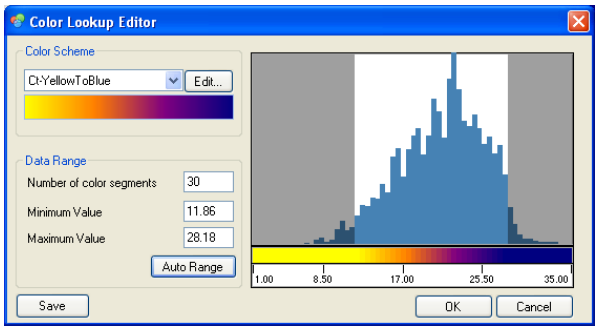


2 Change the values.

In the example below, the minimum and maximum values have been changed to 3.0. Therefore, after discarding the lower and upper 3% of valid values, you are left with a range of 11.86 to 28.18.



This range is represented in the **Color Lookup Editor** illustrated below. Note the eliminated values (from 1 to 11.86, and 28.18 to 35) are now gray areas.



3 Click **OK** to see the changes in the heat map and the heat map legend.

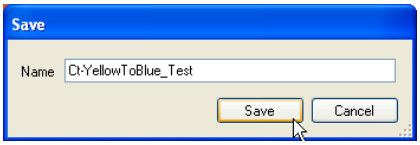
Saving Changes

To save custom parameters that you have set:

1 Click **Save**.

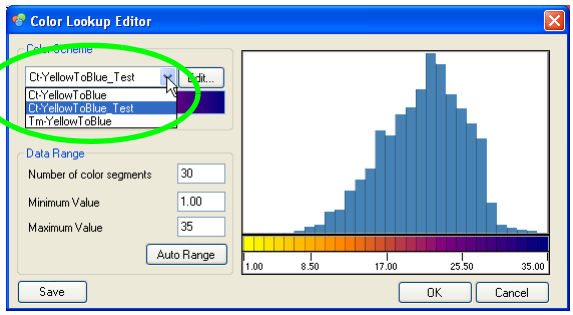
2 Enter a name for your custom parameters.

3 Click **Save**.



The **Color Lookup Editor** opens.

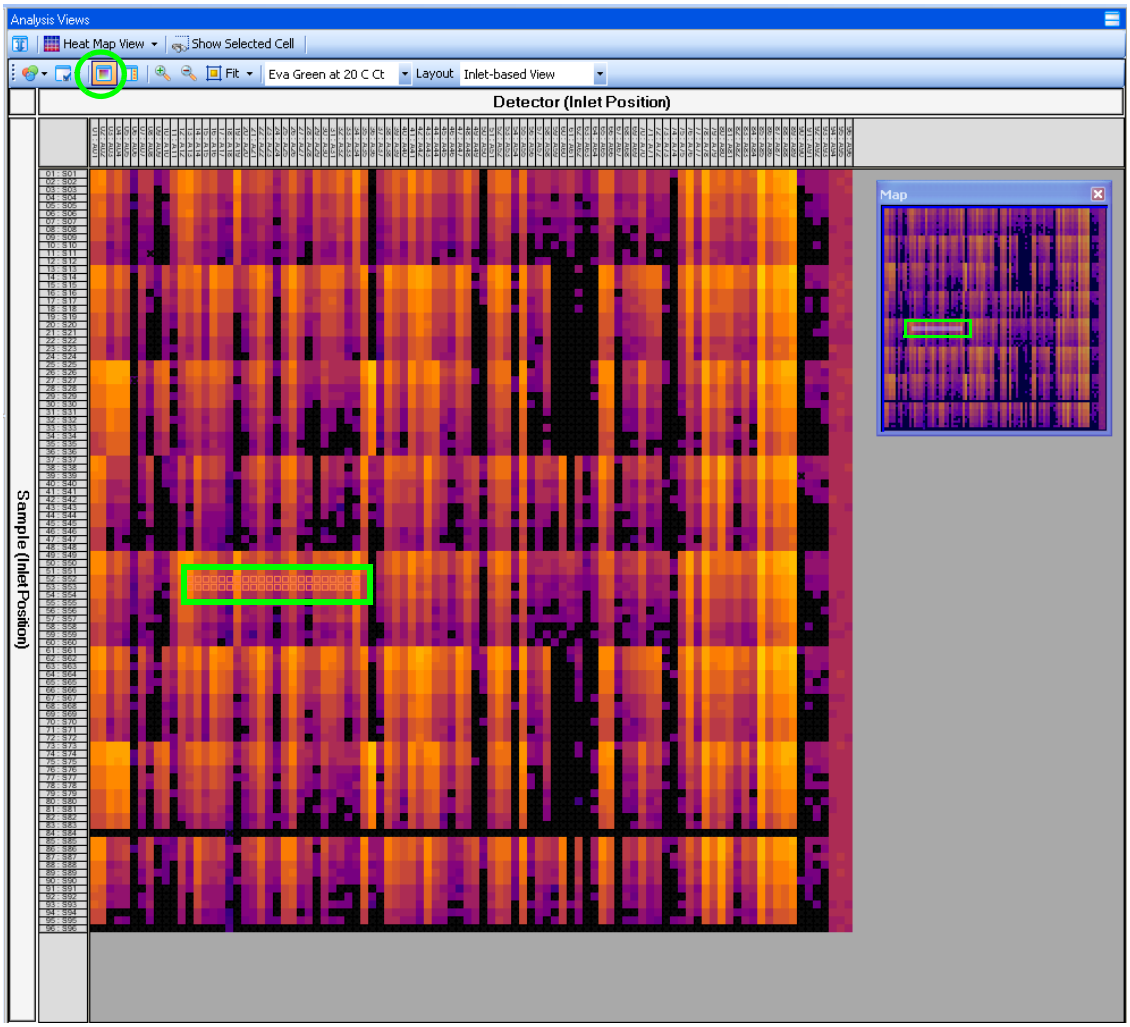
4 Click the **Color Scheme** menu to see the saved parameters.



Location Reference Map

Use the location map to reference your cell of interest within the entire framework of the chip.

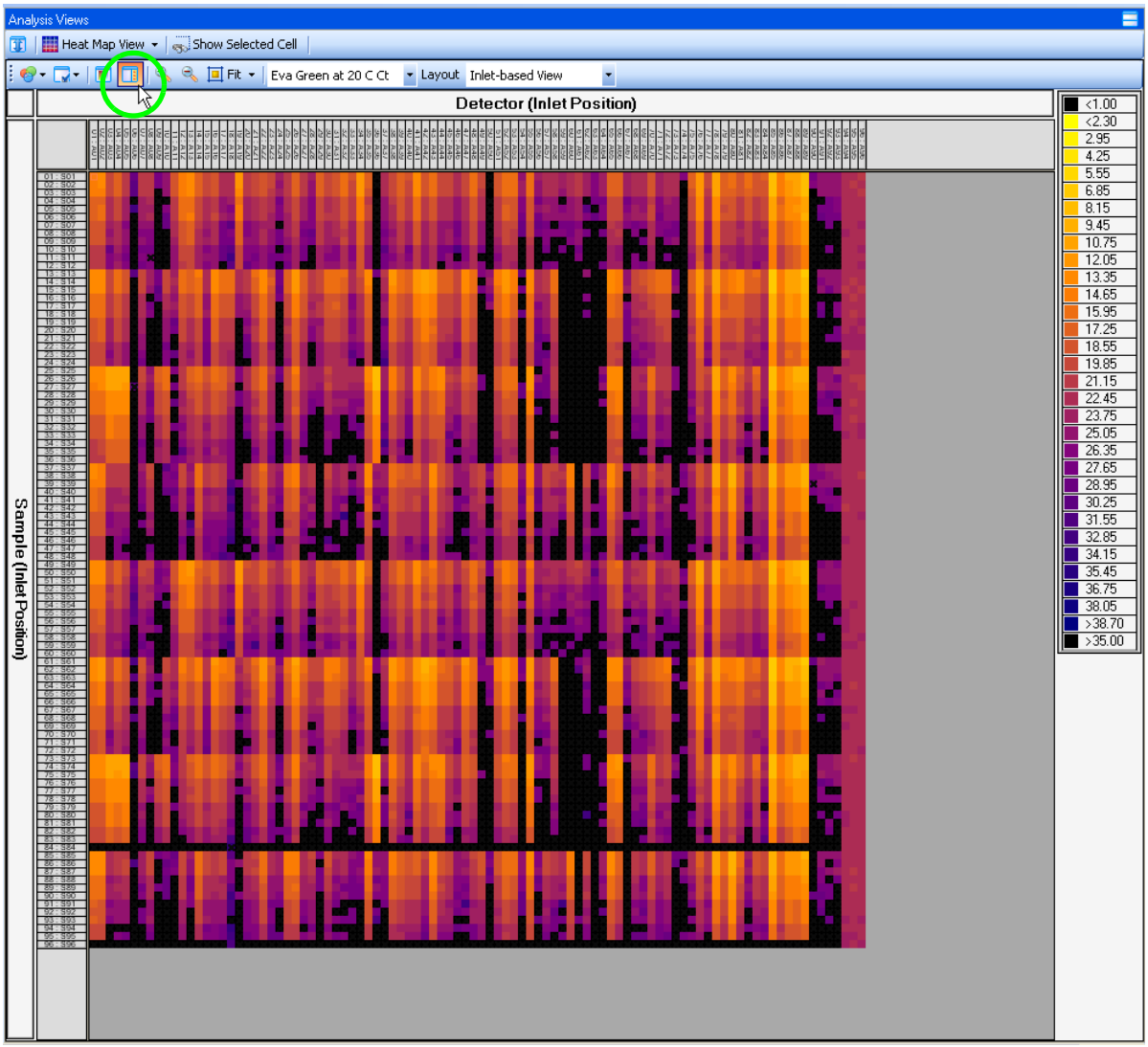
- Click the **Location Reference map** icon to open the map.



Legend

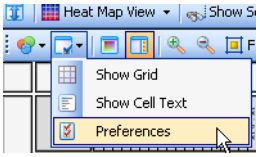
The legend is a color representation of the C_t or T_m values displayed on the heat map.

- Click the **Legend** icon .



Preferences

Click the **Preferences** button .

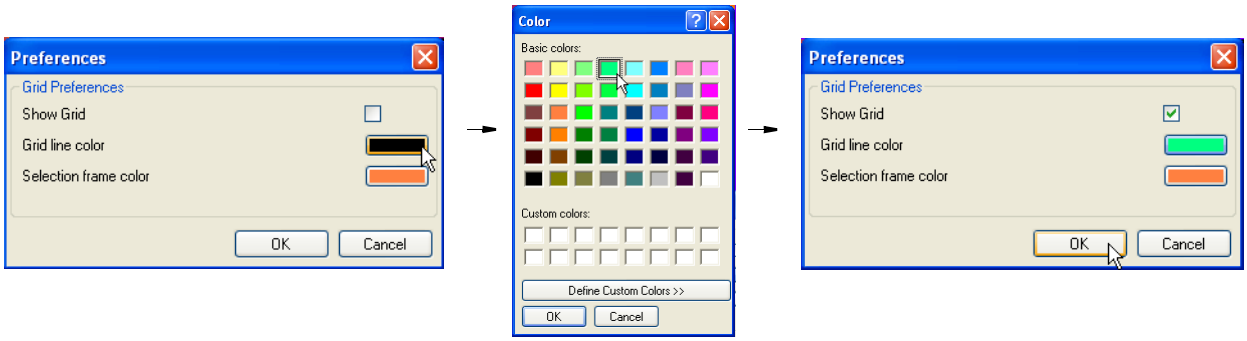


- Show Grid
- Show Cell Text
- Preferences

Changing Grid and Selected Cell Color Preferences

To change heat map grid lines:

1. Click **Preferences**.
2. Click the **Grid line color** rectangle.
   The color palette opens.

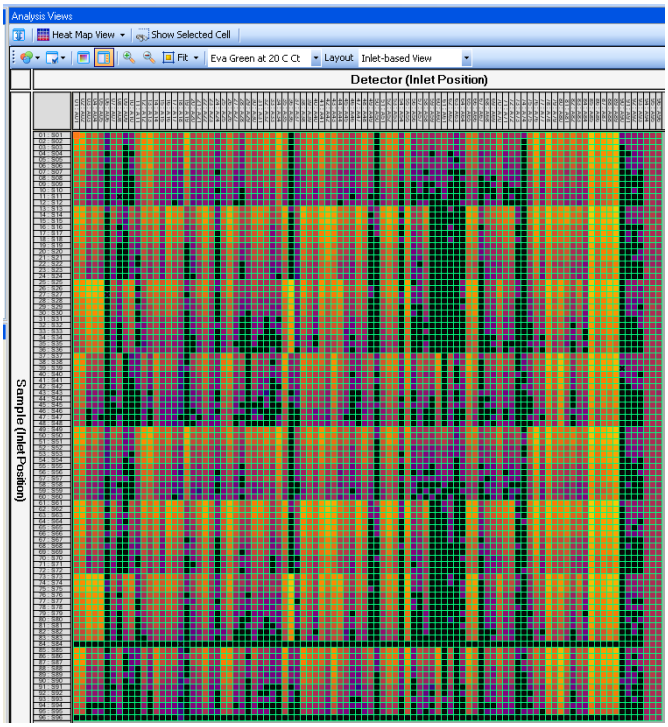


3 Click on a color.

4 Click **OK**.

5 Click the **Show Grid** box.

6 Click **OK**. The new color grid lines display in the heat map.



7 *Optional:* Click **Show Grid** again to toggle the grid on and off.

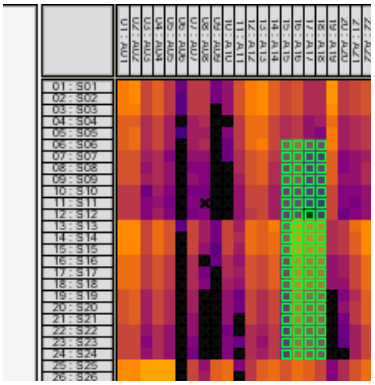
To change the color of the borders of selected cells:

1 Click the **Preferences** button .

2 Click the **Selection frame color** rectangle.
The color palette opens.

3 Click a color.

4 Click **OK**. Frames of selected cells now show the new color in the heat map.

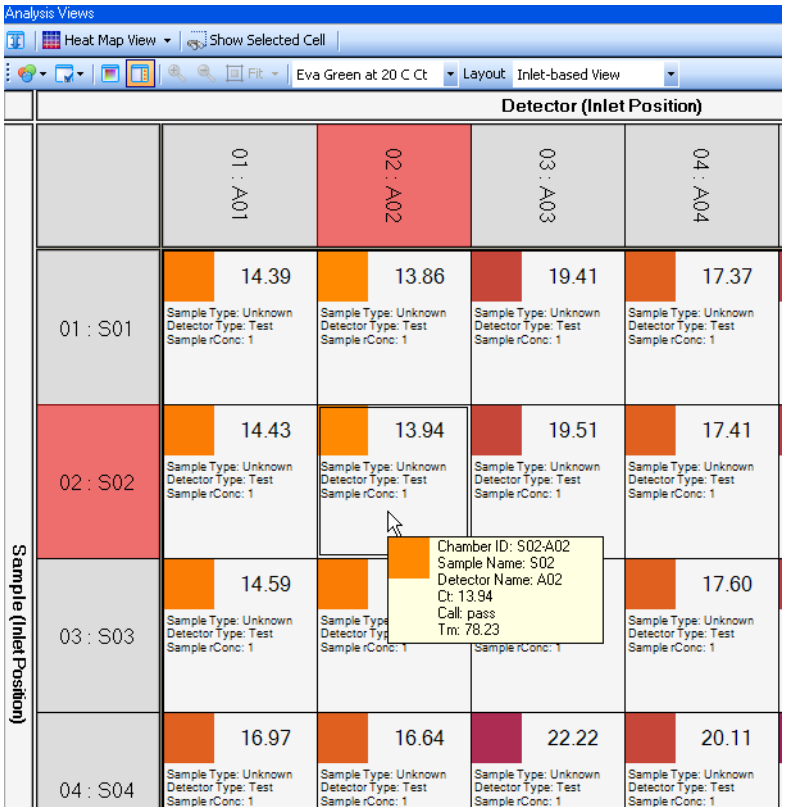


Selected cells in green

Show Cell Text

To show the details of a cell in text:

1. Click **Preferences**.
2. Click **Show Cell Text**. Heat map cells are enlarged and text is now visible.



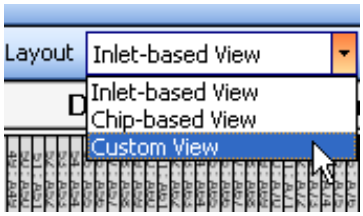
3. *Optional:* Click **Show Cell Text** again to toggle the text view on and off.

Zoom

Increase or decrease the image view size by multi-clicking the magnifying glass icons (+ and –)

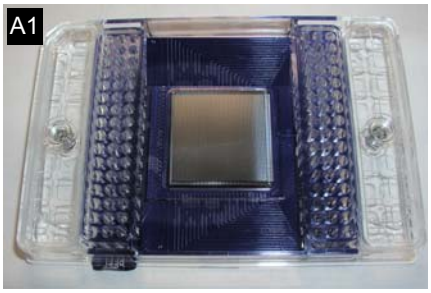
Layout View

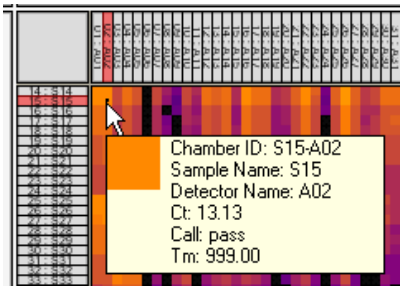
Toggle between inlet-based, chip-based, and custom views in the heat map.



Inlet-Based View

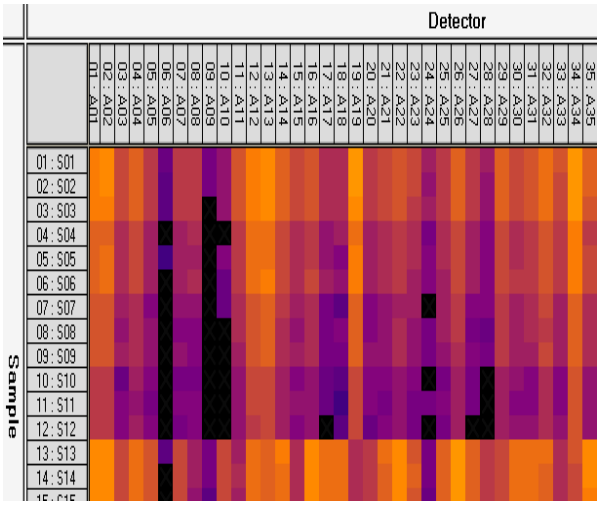
The inlet-based view shows the cell in the same numbered sequence as the inlets on the chip, as shown below.





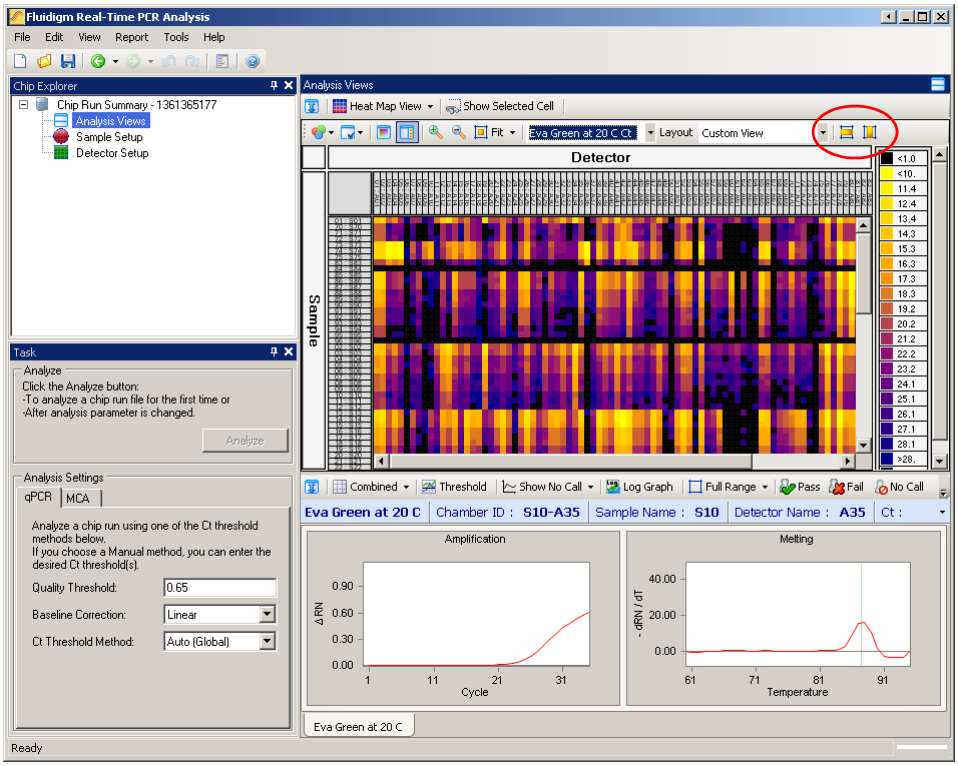
Chip-Based View

The chip-based view shows a sequence of numbers assigned to chambers on a chip counting from top-left corner to the right and then, top to bottom.

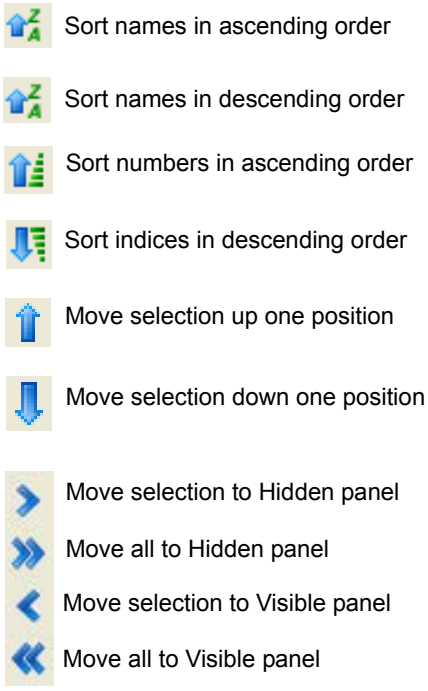


Custom View

The Custom View selection adds two buttons to the tool bar, one to modify the column layout and one to modify the row format. Both function in the same manner (see detailed description below). Any modifications are rendered immediately in the heat map. The changes will be saved with the chip run.



From within the B dialog, customize the heat map layout by changing the way rows and columns are displayed. Use the graphic below as a guide.



Sort names in ascending order

Sort names in descending order

Sort numbers in ascending order

Sort indices in descending order

Move selection up one position

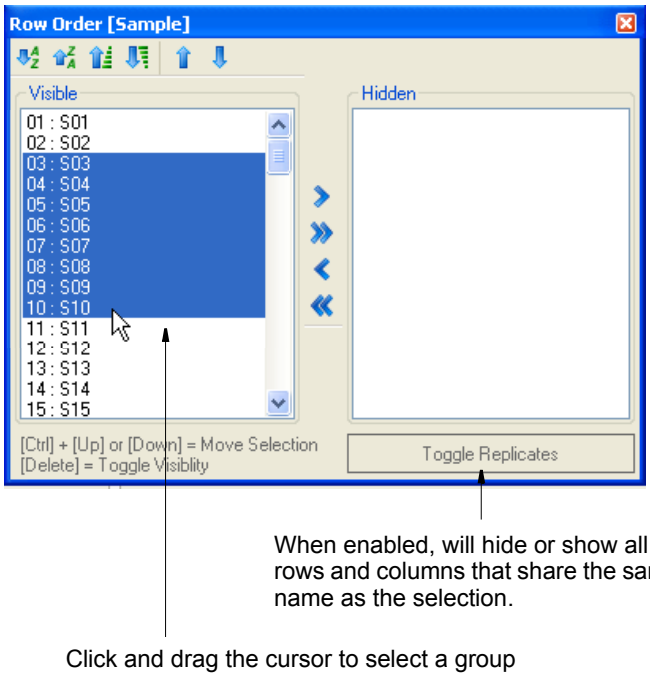
Move selection down one position

Move selection to Hidden panel

Move all to Hidden panel

Move selection to Visible panel

Move all to Visible panel



When enabled, will hide or show all rows and columns that share the same name as the selection.

Click and drag the cursor to select a group

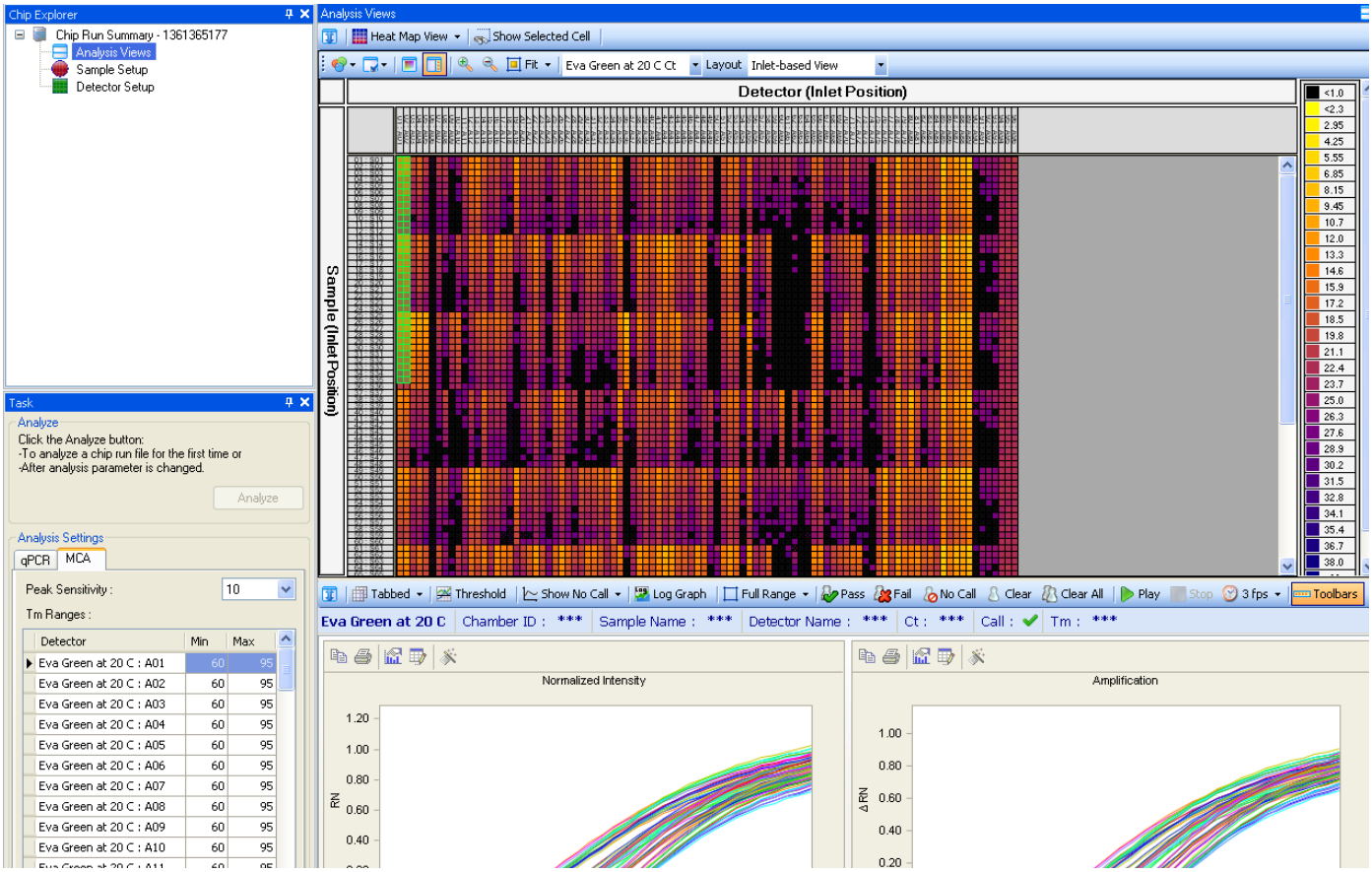
To change row order:

1 Click the **row order button**. The **Row Order [Sample]** dialog opens.

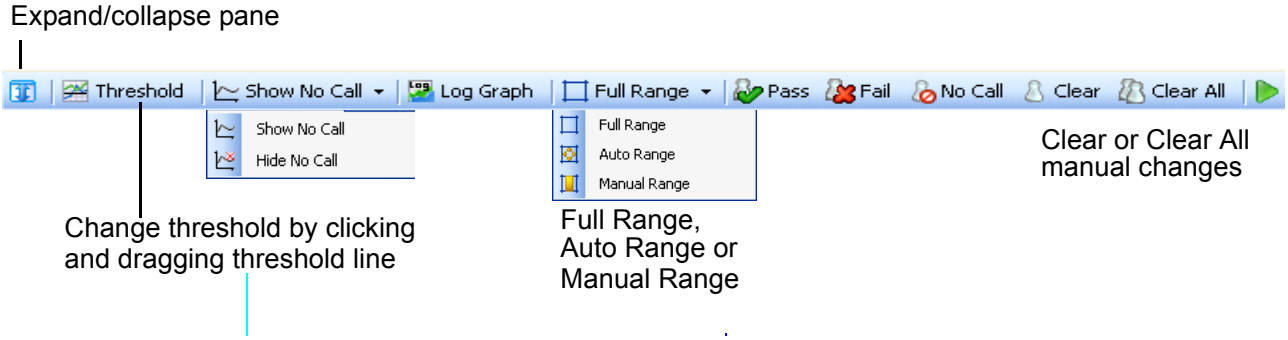
2 Group, reorder, and hide rows and columns as needed.

Using the Graph View

The graph view displays the curve data and information about the current selection of chambers. The secondary tool bar allows you to change the display of the data.

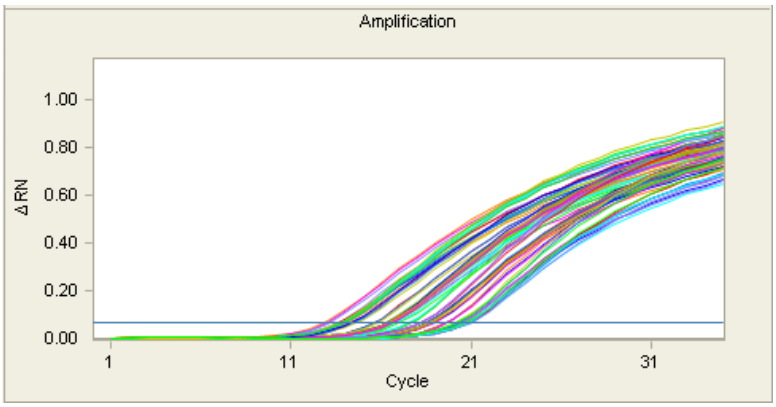


Graph Viewer Tool Bar



Toggling the Threshold

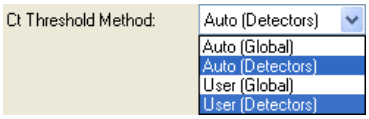
Click the **Threshold** button to apply a C_t threshold line to the amplification graph.



With threshold baseline

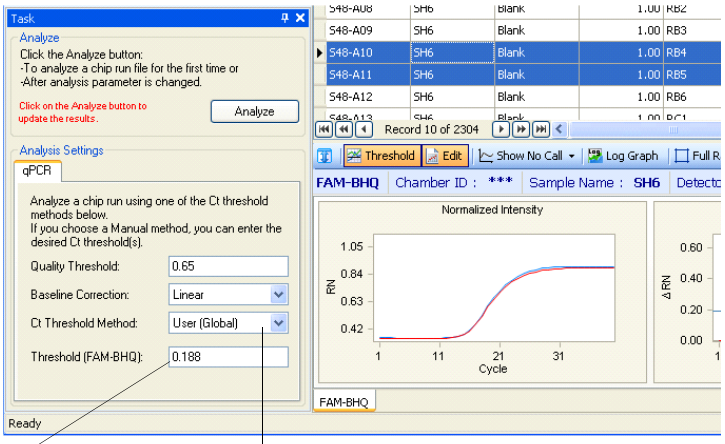
Using the Graph Edit Button

IMPORTANT: The **Edit** button is enabled only when the **CT Threshold Method** is either **Auto (Detectors)** or **User (Detectors)**.



Toggle Edit

Click **Toggle Edit** in conjunction with Toggle Threshold enable moving the threshold bar to a new position by clicking and dragging it in the lower graph. This can only be done in **User Data Global** or **User Data Detector** threshold analysis methods.

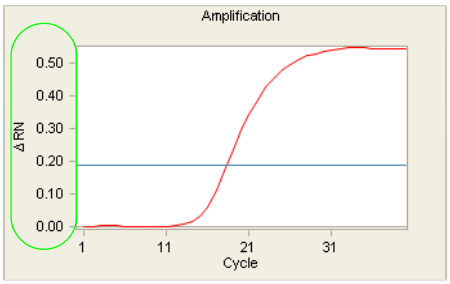


Threshold number changes as you re-position the threshold line.

C_t Threshold Method can be only User Data (Global) or User (Data Detectors) for Toggle Edit to be enabled.

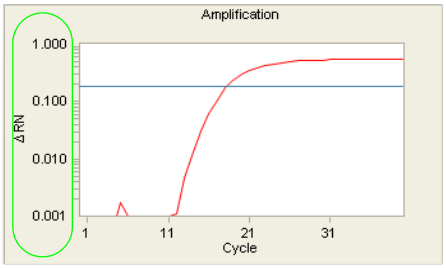
Toggle Log Graph

The log graph shows more detail of the same view. Note the finer scale on the log graph *y* axis below left.



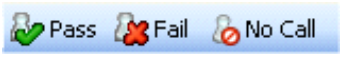
Log graph *off*

Log graph *on*

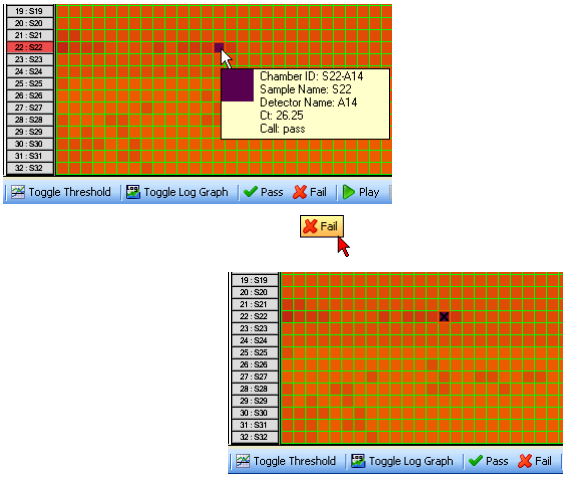


Changing Pass/Fail

If the heat map reveals a problematic experiment, you can manually change the call to exclude the experiment. Change cells to pass or fail as appropriate. After reviwing the data, you can manually change the call to **Pass**, **Fail** or **No Call**. **No Call** indicates that the data in the chamber is determined to be invalid and should not be considered in further calculations. In the example below, the passing cell is manually failed.

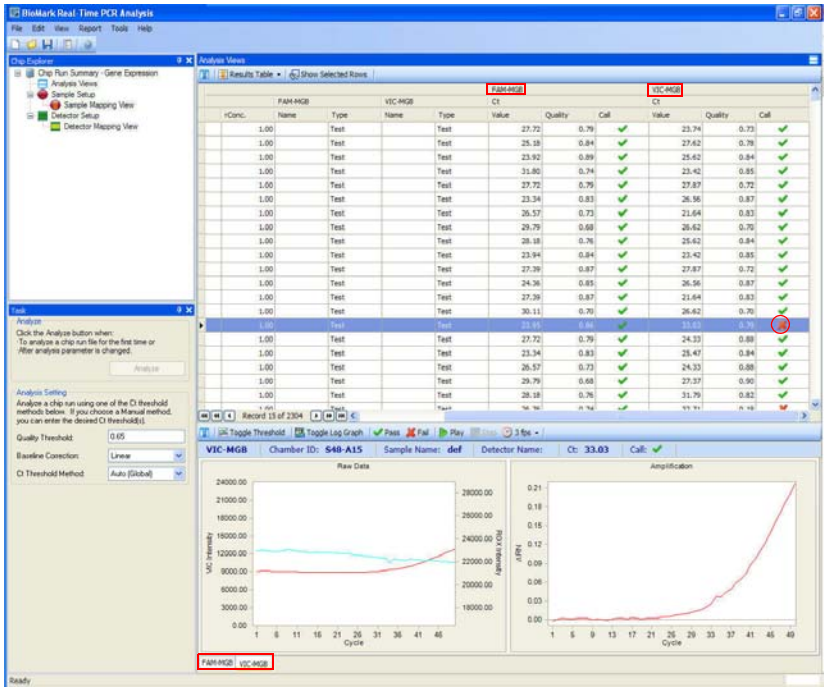


1. Click a cell to activate it.
2. Click the **Pass** or the **Fail** icon. Or, click **Edit > Pass/Fail**.



If you used two probes, make sure the appropriate graph tab is active. In the example below, FAM-MGB and VIC-MGB probes each have a tab on the graph view. Click the appropriate graph tab before changing the call.

Probe-specific column heads



Probe-specific graph tabs

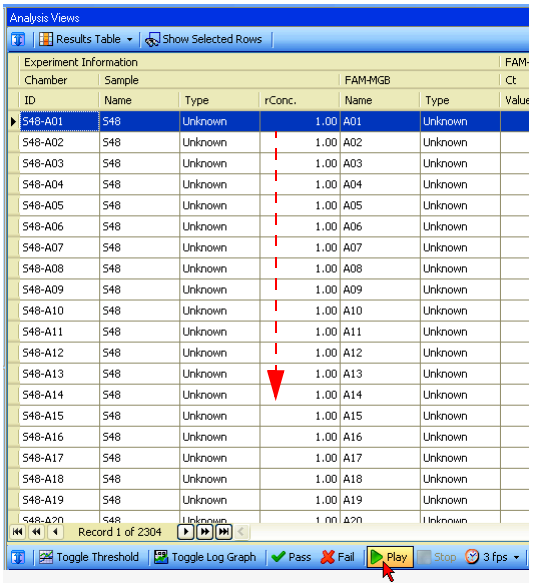
NOTE: You can use the **Call Redo** or **Undo** buttons to revert back to the original call state.

Using the Animate Feature

In the Graph Views, watch an animation of each cell on the entire chip in sequence. Use this feature while in the **Results Table**, **Image View**, and/or **Heat Map**.

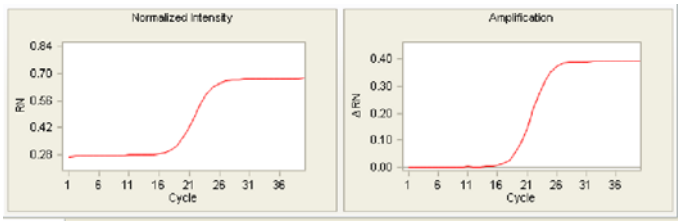
1. Click a cell or row.
2. Click **Play**.

   Watch the Normalized Intensity and Amplification graphs as each cell is displayed in sequence.



Click a row (in the Results Table in this example) and then click *Play*.

The highlighted row is displayed in the graph views and then the animation jumps to the next cell in sequence and so on until you click Stop or, the animation runs through the entire chip.



Each cell's data are displayed in the graphs during the animation.

3 *Optional:* Adjust the animation speed.

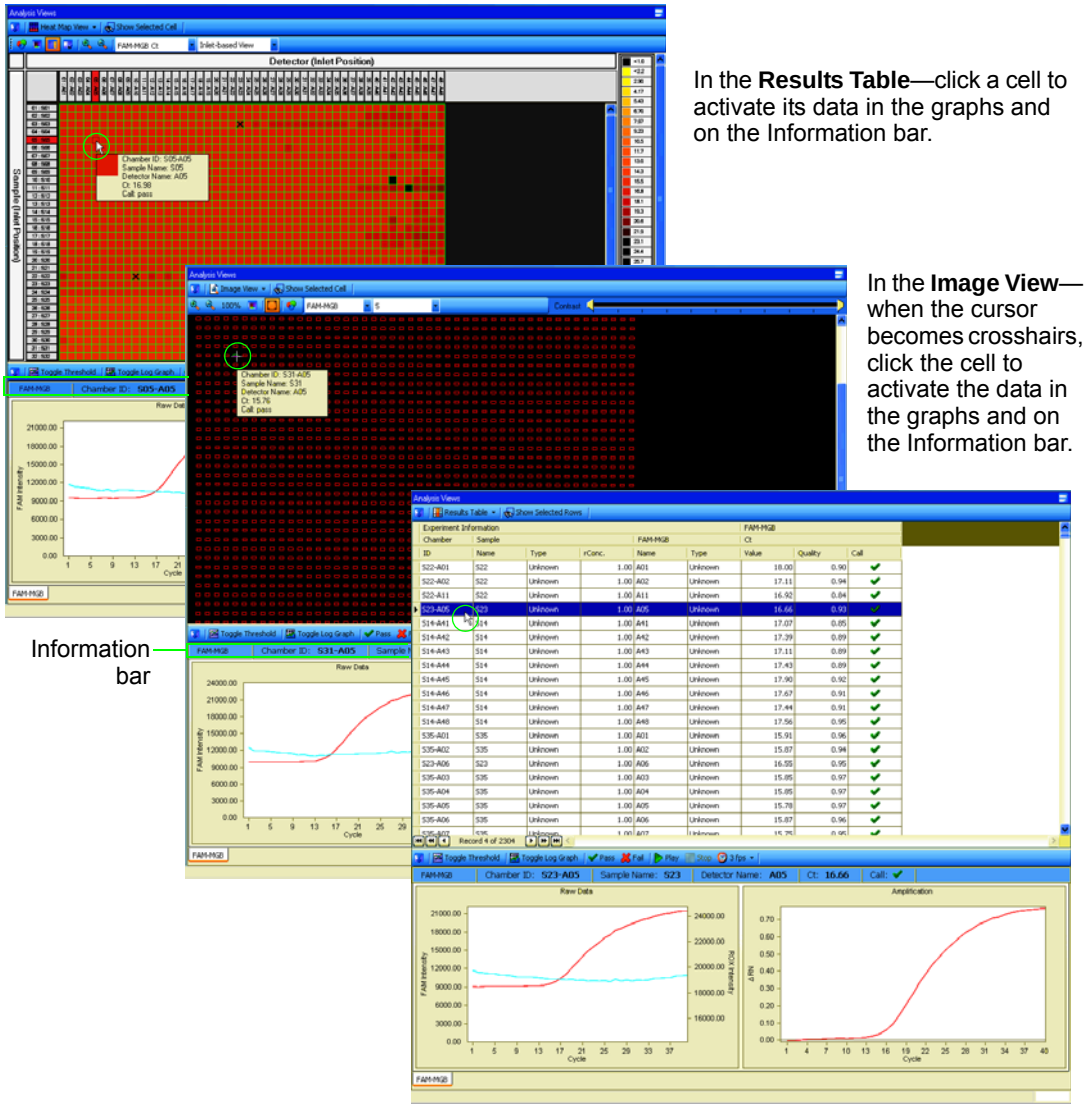
a Click .

b Choose a viewing speed.

4 Click **Stop** to stop the animation.

5 Click **Play** to continue the animation.

Selecting a Single Cell



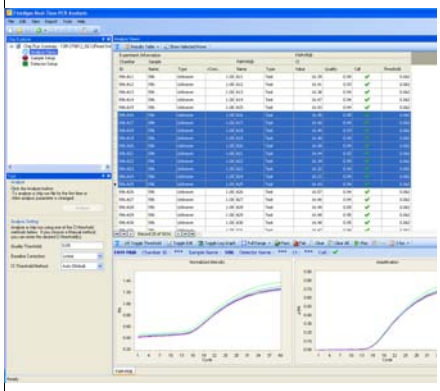
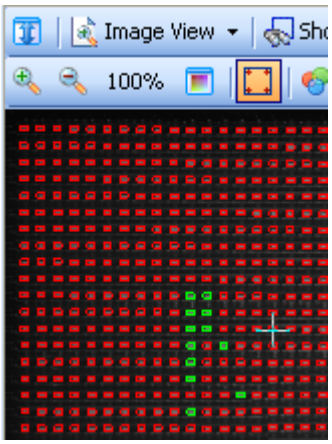
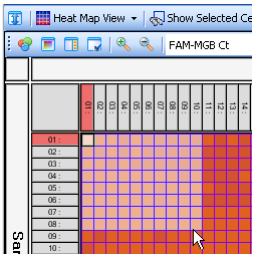
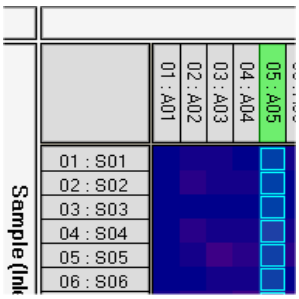
In the **Results Table**—click a cell to activate its data in the graphs and on the Information bar.

In the **Image View**—when the cursor becomes crosshairs, click the cell to activate the data in the graphs and on the Information bar.

In the **Heat Map**—click a cell to activate its data in the graphs and on the Information bar.

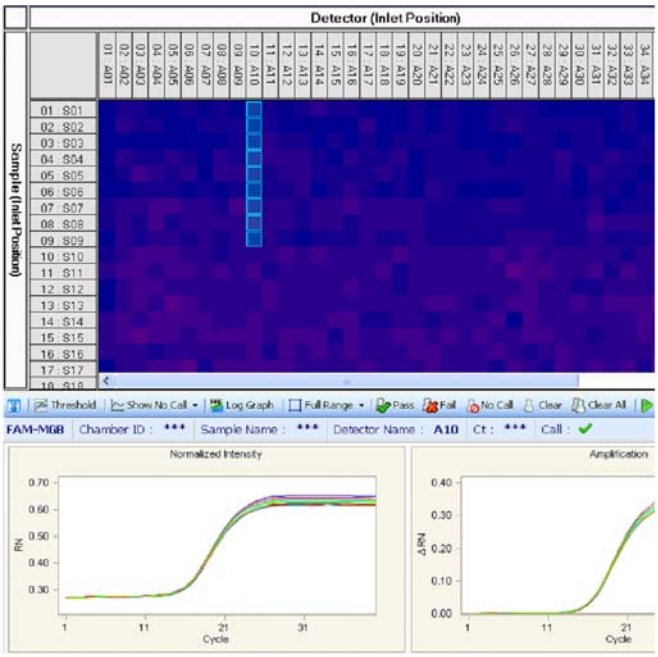
Selecting More Than One Cell

Isolate data for a single cell or for multiple cells in any analysis view (Results Table, Image View, or Heat Map) using the following methods

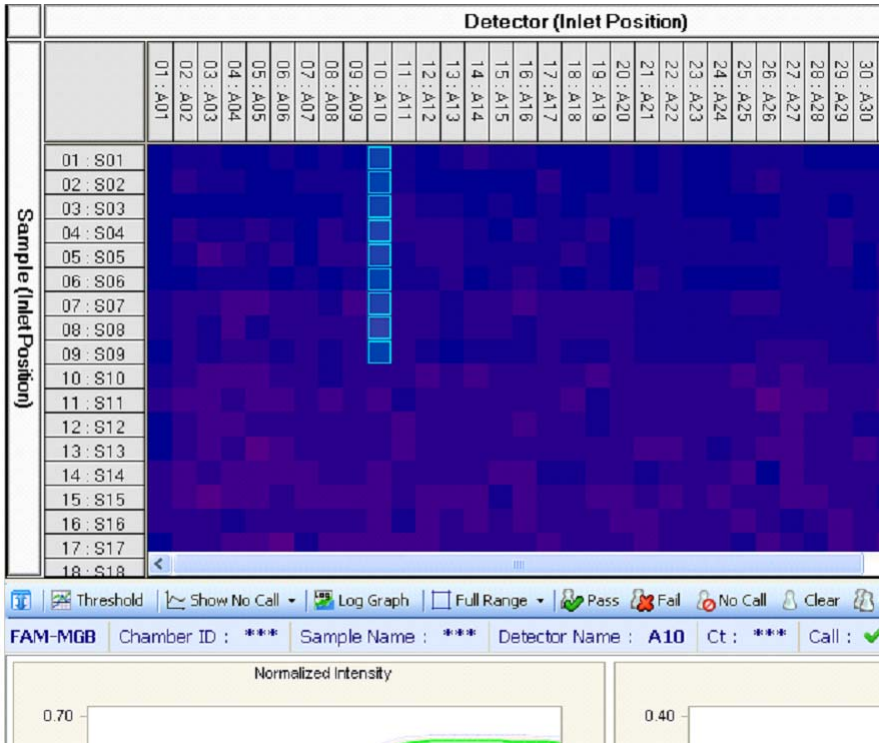
In the Analysis Views Window	Procedure	Example
The Results Table	Press and hold the keyboard **SHIFT** key and click the 2 outer cells for a continuous range of cells. The data for the range of cells display in the Graph Views. or, Press and hold the keyboard **CTRL** key while clicking individual cells.	
Image View	Press and hold the keyboard **CTRL** key while clicking individual cells of interest. You cannot select a contiguous range in this view.	
Heat Map	Click a cell and then hold and drag to highlight a range of cells. Or, Press and hold the keyboard **CTRL** key while clicking individual cells. Or, Click on the Column or Row heading and select all chambers in the column or row.	 

Cross Highlighting and Selecting

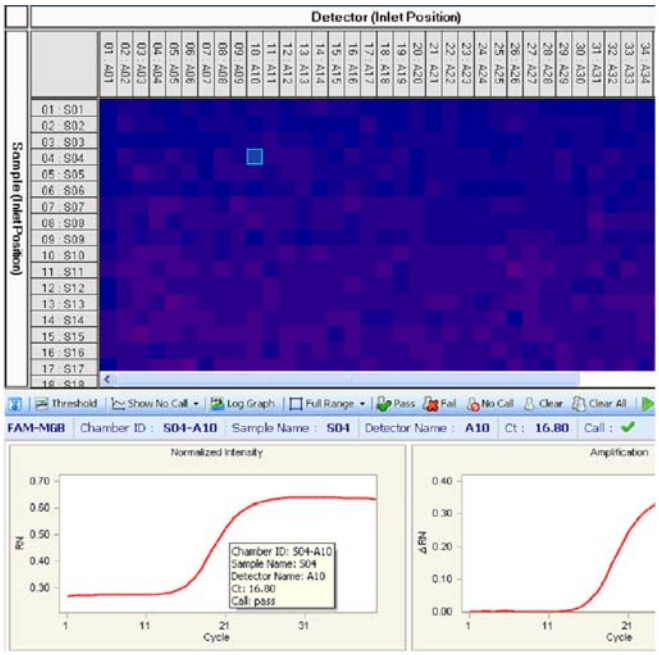
When you select multiple chambers, they are displayed as a line in the lower graphs.



To Cross Highlight: hover the mouse over a graph line and its chamber will be highlighted in the other graphs.



Double-clicking on the graph line selects it in the primary view and the graph view.



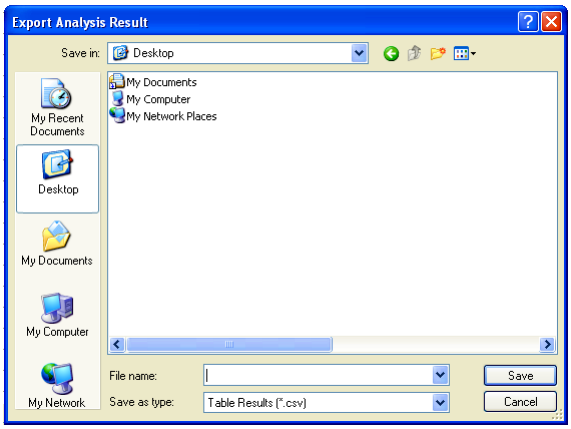
Exporting Data

You can export analysis data from the Heat Map and the Results Table views. The data are exported as Comma Separated Values (.csv) files that can be opened in Microsoft Excel.

NOTE: The data look different in the .csv file depending on the view from which you exported.

Exporting Data from the Results Table

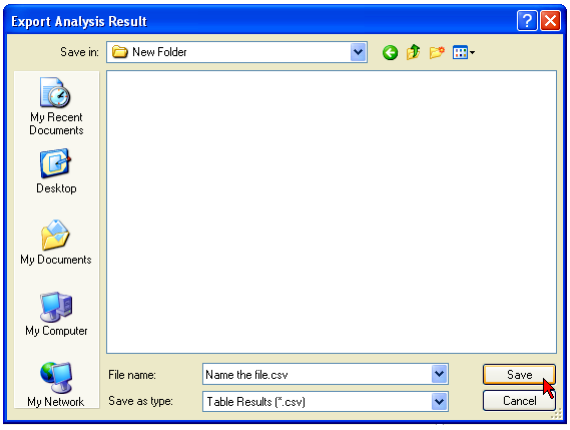
1 While in the **Results Table**, click the **Export** icon or go to **File > Export**. The **Export Analysis Result** dialog opens.



2 Name the .csv file (that is, the data you are exporting).

3 Navigate to the save location.

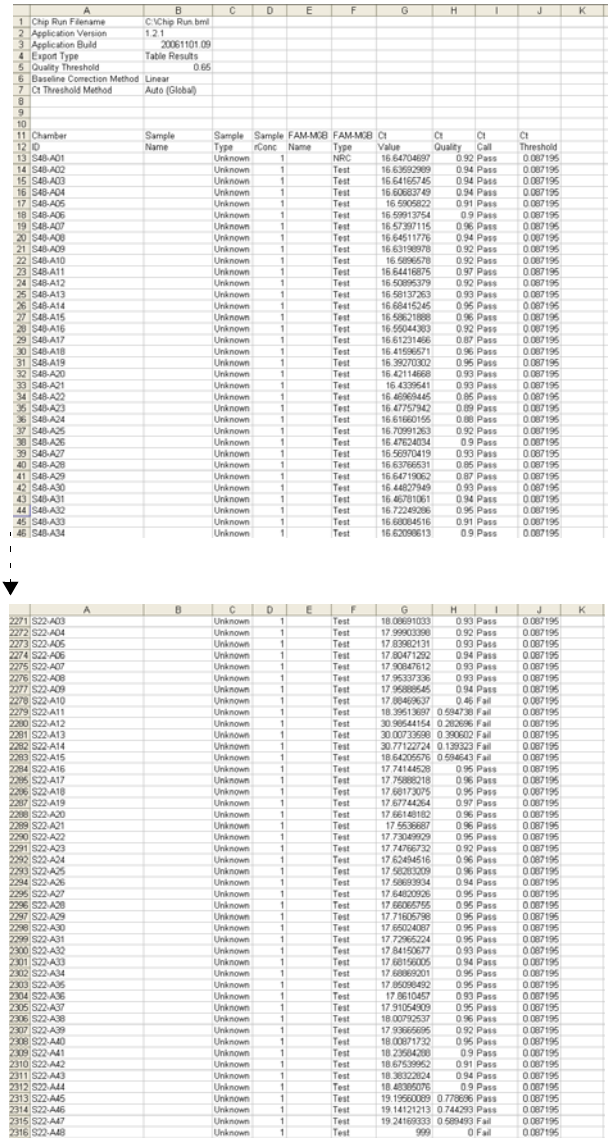
4 Click **Save** when you are at the save location.



Opening Exported Data (.csv files)

1 Double-click the saved .csv file of interest.

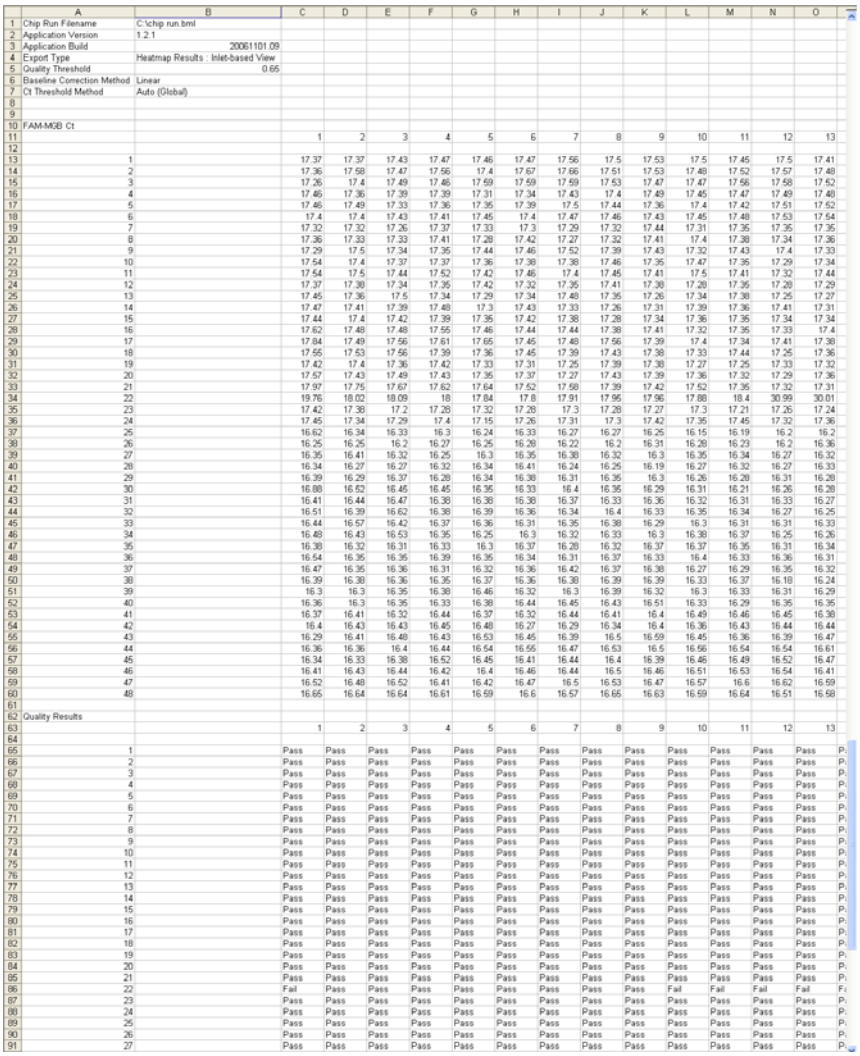
The exported .csv file below was saved from the **Results Table** view.



	A	B	C	D	E	F	G	H	I	J	K
1	Chip Run Filename	C:\Chip Run.bml									
2	Application Version	1.2.1									
3	Application Build	20061101.09									
4	Export Type	Table Results									
5	Quality Threshold	0.65									
6	Baseline Correction Method	Linear									
7	Ct Threshold Method	Auto (Global)									
8											
9											
10											
11	Chamber	Sample	Sample	Sample	FAM-MGB	FAM-MGB	Ct	Ct	Ct	Ct	
12	ID	Name	Type	rConc	Name	Type	Value	Quality	Call	Threshold	
13	S48-A01		Unknown	1		NRC	16.64704697	0.92	Pass	0.087195	
14	S48-A02		Unknown	1		Test	16.63592989	0.94	Pass	0.087195	
15	S48-A03		Unknown	1		Test	16.64165745	0.94	Pass	0.087195	
16	S48-A04		Unknown	1		Test	16.60683749	0.94	Pass	0.087195	
17	S48-A05		Unknown	1		Test	16.5905822	0.91	Pass	0.087195	
18	S48-A06		Unknown	1		Test	16.59913754	0.9	Pass	0.087195	
19	S48-A07		Unknown	1		Test	16.57397115	0.96	Pass	0.087195	
20	S48-A08		Unknown	1		Test	16.64511776	0.94	Pass	0.087195	
21	S48-A09		Unknown	1		Test	16.63198978	0.92	Pass	0.087195	
22	S48-A10		Unknown	1		Test	16.5896578	0.92	Pass	0.087195	
23	S48-A11		Unknown	1		Test	16.64416876	0.97	Pass	0.087195	
24	S48-A12		Unknown	1		Test	16.50895379	0.92	Pass	0.087195	
25	S48-A13		Unknown	1		Test	16.58137263	0.93	Pass	0.087195	
26	S48-A14		Unknown	1		Test	16.68415245	0.95	Pass	0.087195	
27	S48-A15		Unknown	1		Test	16.59521888	0.96	Pass	0.087195	
28	S48-A16		Unknown	1		Test	16.55044383	0.92	Pass	0.087195	
29	S48-A17		Unknown	1		Test	16.61231466	0.87	Pass	0.087195	
30	S48-A18		Unknown	1		Test	16.41596571	0.96	Pass	0.087195	
31	S48-A19		Unknown	1		Test	16.39270302	0.95	Pass	0.087195	
32	S48-A20		Unknown	1		Test	16.42114668	0.93	Pass	0.087195	
33	S48-A21		Unknown	1		Test	16.4339541	0.93	Pass	0.087195	
34	S48-A22		Unknown	1		Test	16.46969445	0.85	Pass	0.087195	
35	S48-A23		Unknown	1		Test	16.47757942	0.89	Pass	0.087195	
36	S48-A24		Unknown	1		Test	16.61660155	0.88	Pass	0.087195	
37	S48-A25		Unknown	1		Test	16.70991263	0.92	Pass	0.087195	
38	S48-A26		Unknown	1		Test	16.47624034	0.9	Pass	0.087195	
39	S48-A27		Unknown	1		Test	16.56970419	0.93	Pass	0.087195	
40	S48-A28		Unknown	1		Test	16.63766531	0.85	Pass	0.087195	
41	S48-A29		Unknown	1		Test	16.64719062	0.87	Pass	0.087195	
42	S48-A30		Unknown	1		Test	16.44827949	0.93	Pass	0.087195	
43	S48-A31		Unknown	1		Test	16.46781061	0.94	Pass	0.087195	
44	S48-A32		Unknown	1		Test	16.72249286	0.95	Pass	0.087195	
45	S48-A33		Unknown	1		Test	16.68084516	0.91	Pass	0.087195	
46	S48-A34		Unknown	1		Test	16.62098613	0.9	Pass	0.087195	

	A	B	C	D	E	F	G	H	I	J	K
2271	S22-A03		Unknown	1		Test	18.08691033	0.93	Pass	0.087195	
2272	S22-A04		Unknown	1		Test	17.99903398	0.92	Pass	0.087195	
2273	S22-A05		Unknown	1		Test	17.83982131	0.93	Pass	0.087195	
2274	S22-A06		Unknown	1		Test	17.80471292	0.94	Pass	0.087195	
2275	S22-A07		Unknown	1		Test	17.90847612	0.93	Pass	0.087195	
2276	S22-A08		Unknown	1		Test	17.95337336	0.93	Pass	0.087195	
2277	S22-A09		Unknown	1		Test	17.95888545	0.94	Pass	0.087195	
2278	S22-A10		Unknown	1		Test	17.88469637	0.46	Fail	0.087195	
2279	S22-A11		Unknown	1		Test	18.39513697	0.594738	Fail	0.087195	
2280	S22-A12		Unknown	1		Test	30.96544154	0.262696	Fail	0.087195	
2281	S22-A13		Unknown	1		Test	30.00733598	0.390602	Fail	0.087195	
2282	S22-A14		Unknown	1		Test	30.77122724	0.139323	Fail	0.087195	
2283	S22-A15		Unknown	1		Test	18.64205576	0.594643	Fail	0.087195	
2284	S22-A16		Unknown	1		Test	17.74144528	0.95	Pass	0.087195	
2285	S22-A17		Unknown	1		Test	17.75888218	0.96	Pass	0.087195	
2286	S22-A18		Unknown	1		Test	17.68173075	0.95	Pass	0.087195	
2287	S22-A19		Unknown	1		Test	17.67744264	0.97	Pass	0.087195	
2288	S22-A20		Unknown	1		Test	17.66148182	0.96	Pass	0.087195	
2289	S22-A21		Unknown	1		Test	17.5538687	0.96	Pass	0.087195	
2290	S22-A22		Unknown	1		Test	17.73049929	0.95	Pass	0.087195	
2291	S22-A23		Unknown	1		Test	17.74766732	0.92	Pass	0.087195	
2292	S22-A24		Unknown	1		Test	17.62494516	0.96	Pass	0.087195	
2293	S22-A25		Unknown	1		Test	17.58283209	0.96	Pass	0.087195	
2294	S22-A26		Unknown	1		Test	17.58693934	0.94	Pass	0.087195	
2295	S22-A27		Unknown	1		Test	17.64820926	0.95	Pass	0.087195	
2296	S22-A28		Unknown	1		Test	17.66066755	0.96	Pass	0.087195	
2297	S22-A29		Unknown	1		Test	17.71605798	0.96	Pass	0.087195	
2298	S22-A30		Unknown	1		Test	17.65024087	0.95	Pass	0.087195	
2299	S22-A31		Unknown	1		Test	17.72965224	0.95	Pass	0.087195	
2300	S22-A32		Unknown	1		Test	17.84150677	0.93	Pass	0.087195	
2301	S22-A33		Unknown	1		Test	17.68156005	0.94	Pass	0.087195	
2302	S22-A34		Unknown	1		Test	17.68869201	0.95	Pass	0.087195	
2303	S22-A35		Unknown	1		Test	17.85098492	0.95	Pass	0.087195	
2304	S22-A36		Unknown	1		Test	17.8610457	0.93	Pass	0.087195	
2305	S22-A37		Unknown	1		Test	17.91054909	0.95	Pass	0.087195	
2306	S22-A38		Unknown	1		Test	18.00792537	0.96	Pass	0.087195	
2307	S22-A39		Unknown	1		Test	17.93666695	0.92	Pass	0.087195	
2308	S22-A40		Unknown	1		Test	18.00871732	0.95	Pass	0.087195	
2309	S22-A41		Unknown	1		Test	18.23584288	0.9	Pass	0.087195	
2310	S22-A42		Unknown	1		Test	18.67539952	0.91	Pass	0.087195	
2311	S22-A43		Unknown	1		Test	18.38322824	0.94	Pass	0.087195	
2312	S22-A44		Unknown	1		Test	18.48386076	0.9	Pass	0.087195	
2313	S22-A45		Unknown	1		Test	19.19560089	0.778696	Pass	0.087195	
2314	S22-A46		Unknown	1		Test	19.14121213	0.744293	Pass	0.087195	
2315	S22-A47		Unknown	1		Test	19.24169333	0.589493	Fail	0.087195	
2316	S22-A48		Unknown	1		Test	999	0	Fail	0.087195	

The exported .csv file below was saved from the **Heat Map** view.

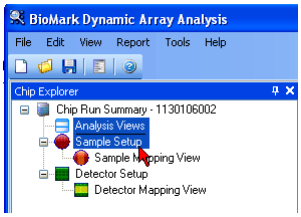


	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Chip Run Filename	C:\chip run.bml													
2	Application Version	1.2.1													
3	Application Build	20061101.09													
4	Export Type	Heatmap Results : Inlet-based View													
5	Quality Threshold	0.65													
6	Baseline Correction Method	Linear													
7	Ct Threshold Method	Auto (Global)													
8															
9															
10	FAM-MGB Ct														
11			1	2	3	4	5	6	7	8	9	10	11	12	13
12															
13		1	17.37	17.37	17.43	17.47	17.46	17.47	17.56	17.5	17.53	17.5	17.45	17.5	17.41
14		2	17.36	17.58	17.47	17.56	17.4	17.67	17.66	17.51	17.53	17.48	17.52	17.57	17.48
15		3	17.26	17.4	17.49	17.46	17.69	17.69	17.69	17.53	17.47	17.47	17.66	17.68	17.52
16		4	17.46	17.36	17.39	17.39	17.31	17.34	17.43	17.4	17.49	17.45	17.47	17.49	17.48
17		5	17.46	17.49	17.33	17.36	17.35	17.39	17.5	17.44	17.36	17.4	17.42	17.51	17.52
18		6	17.4	17.4	17.43	17.41	17.45	17.4	17.47	17.46	17.43	17.45	17.48	17.53	17.54
19		7	17.32	17.32	17.26	17.37	17.33	17.3	17.29	17.32	17.44	17.31	17.35	17.35	17.35
20		8	17.36	17.33	17.33	17.41	17.28	17.42	17.27	17.32	17.41	17.4	17.38	17.34	17.36
21		9	17.29	17.5	17.34	17.35	17.44	17.46	17.52	17.39	17.43	17.32	17.43	17.4	17.33
22		10	17.54	17.4	17.37	17.37	17.36	17.38	17.38	17.46	17.35	17.47	17.35	17.29	17.34
23		11	17.54	17.5	17.44	17.52	17.42	17.46	17.4	17.45	17.41	17.5	17.41	17.32	17.44
24		12	17.37	17.38	17.34	17.35	17.42	17.32	17.35	17.41	17.38	17.28	17.35	17.28	17.29
25		13	17.45	17.36	17.5	17.34	17.29	17.34	17.48	17.35	17.26	17.34	17.38	17.25	17.27
26		14	17.47	17.41	17.39	17.49	17.3	17.43	17.33	17.26	17.31	17.39	17.36	17.41	17.31
27		15	17.44	17.4	17.42	17.39	17.35	17.42	17.38	17.28	17.34	17.36	17.35	17.34	17.34
28		16	17.62	17.48	17.48	17.55	17.46	17.44	17.44	17.38	17.41	17.32	17.35	17.33	17.4
29		17	17.84	17.49	17.56	17.61	17.65	17.45	17.48	17.56	17.39	17.4	17.34	17.41	17.38
30		18	17.55	17.53	17.56	17.39	17.36	17.45	17.39	17.43	17.38	17.33	17.44	17.25	17.36
31		19	17.42	17.4	17.36	17.42	17.33	17.31	17.25	17.39	17.38	17.27	17.25	17.33	17.32
32		20	17.57	17.43	17.49	17.43	17.35	17.37	17.27	17.43	17.39	17.36	17.32	17.29	17.36
33		21	17.97	17.75	17.67	17.62	17.64	17.52	17.58	17.39	17.42	17.52	17.35	17.32	17.31
34		22	19.76	18.02	18.09	18	17.84	17.8	17.91	17.96	17.96	17.88	18.4	30.99	30.01
35		23	17.42	17.38	17.2	17.28	17.32	17.28	17.3	17.28	17.27	17.3	17.21	17.26	17.24
36		24	17.45	17.34	17.29	17.4	17.15	17.26	17.31	17.3	17.42	17.35	17.45	17.32	17.36
37		25	16.62	16.34	16.33	16.3	16.24	16.33	16.27	16.27	16.25	16.15	16.19	16.2	16.2
38		26	16.25	16.25	16.2	16.27	16.25	16.28	16.22	16.2	16.31	16.28	16.23	16.2	16.36
39		27	16.35	16.41	16.32	16.25	16.3	16.35	16.38	16.32	16.3	16.35	16.34	16.27	16.32
40		28	16.34	16.27	16.27	16.32	16.34	16.41	16.24	16.25	16.19	16.27	16.32	16.27	16.33
41		29	16.39	16.29	16.37	16.28	16.34	16.38	16.31	16.35	16.3	16.26	16.28	16.31	16.28
42		30	16.88	16.62	16.45	16.45	16.35	16.33	16.4	16.35	16.29	16.31	16.21	16.26	16.28
43		31	16.41	16.44	16.47	16.39	16.39	16.38	16.37	16.33	16.36	16.32	16.31	16.33	16.27
44		32	16.51	16.39	16.62	16.38	16.39	16.36	16.34	16.4	16.33	16.35	16.34	16.27	16.25
45		33	16.44	16.57	16.42	16.37	16.36	16.31	16.35	16.38	16.29	16.3	16.31	16.31	16.33
46		34	16.48	16.43	16.53	16.35	16.25	16.3	16.32	16.33	16.3	16.38	16.37	16.25	16.26
47		35	16.39	16.32	16.31	16.33	16.3	16.37	16.28	16.32	16.37	16.37	16.35	16.31	16.34
48		36	16.54	16.35	16.35	16.39	16.35	16.34	16.31	16.37	16.33	16.4	16.33	16.36	16.31
49		37	16.47	16.35	16.36	16.31	16.32	16.36	16.42	16.37	16.38	16.27	16.29	16.35	16.32
50		38	16.39	16.38	16.36	16.35	16.37	16.36	16.38	16.39	16.39	16.33	16.37	16.18	16.24
51		39	16.3	16.3	16.35	16.38	16.46	16.32	16.3	16.39	16.32	16.3	16.33	16.31	16.29
52		40	16.36	16.3	16.35	16.33	16.38	16.44	16.45	16.43	16.51	16.33	16.29	16.35	16.35
53		41	16.37	16.41	16.32	16.44	16.37	16.32	16.44	16.41	16.4	16.49	16.46	16.45	16.38
54		42	16.4	16.43	16.43	16.45	16.48	16.27	16.29	16.34	16.4	16.36	16.43	16.44	16.44
55		43	16.29	16.41	16.48	16.43	16.53	16.45	16.39	16.5	16.59	16.45	16.36	16.39	16.47
56		44	16.36	16.36	16.4	16.44	16.54	16.55	16.47	16.53	16.5	16.56	16.54	16.54	16.61
57		45	16.34	16.33	16.38	16.52	16.45	16.41	16.44	16.4	16.39	16.46	16.49	16.52	16.47
58		46	16.41	16.43	16.44	16.42	16.4	16.46	16.44	16.5	16.46	16.51	16.53	16.54	16.41
59		47	16.52	16.48	16.52	16.41	16.42	16.47	16.5	16.53	16.47	16.57	16.6	16.62	16.59
60		48	16.65	16.64	16.64	16.61	16.59	16.6	16.57	16.65	16.63	16.59	16.64	16.51	16.58
61															
62	Quality Results														
63			1	2	3	4	5	6	7	8	9	10	11	12	13
64															
65		1	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
66		2	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
67		3	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
68		4	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
69		5	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
70		6	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
71		7	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
72		8	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
73		9	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
74		10	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
75		11	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
76		12	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
77		13	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
78		14	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
79		15	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
80		16	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
81		17	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
82		18	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
83		19	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
84		20	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
85		21	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
86		22	Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Fail	Fail	Fail	Fail
87		23	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
88		24	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
89		25	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
90		26	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
91		27	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass

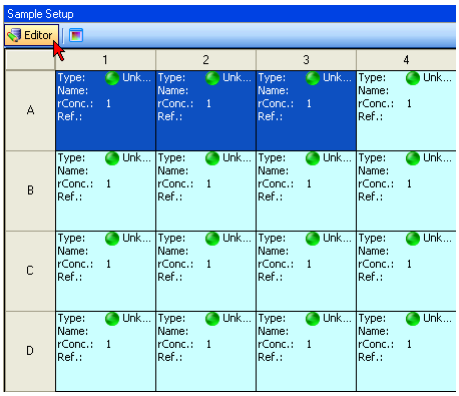
Calculating Delta C_t Sample Values

To calculate the delta (Δ) C_t samples:

1 Click **Sample Setup**.

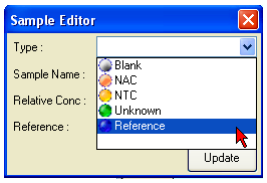


2 Select 1 to 3 cells where the reference sample was added.



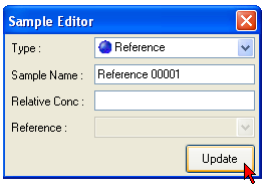
3 Click **Editor**.

4 Click **Reference**.

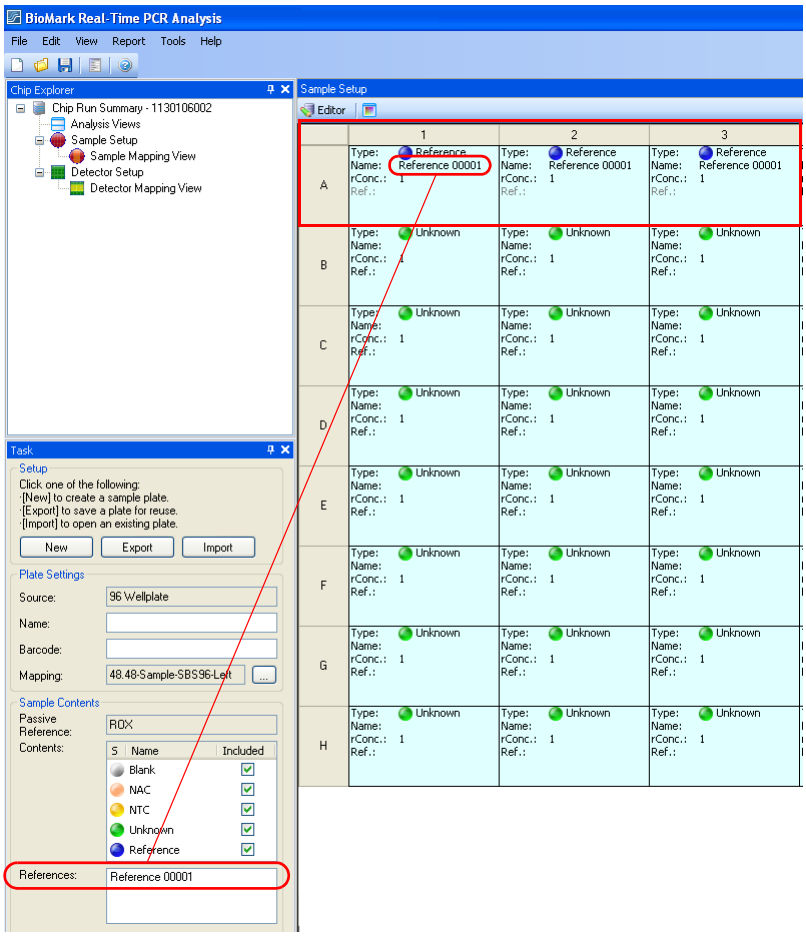


5 Enter a sample name.

6 Click **Update**.



The **Sample Setup** reflects the change.


BioMark Real-Time PCR Analysis
File Edit View Report Tools Help
Chip Explorer
Chip Run Summary - 1130106002
Analysis Views
Sample Setup
Sample Mapping View
Detector Setup
Detector Mapping View
Task
Setup
Click one of the following:
-[New] to create a sample plate.
-[Export] to save a plate for reuse.
-[Import] to open an existing plate.
New
Export
Import
Plate Settings
Source: 96 Wellplate
Name:
Barcode:
Mapping: 48.48-Sample-SBS96-Left
Sample Contents
Passive Reference: ROX
Contents:
S Name Included
Blank
NAC
NTC
Unknown
Reference
References: Reference 00001
Sample Setup
Editor
1
2
3
A
B
C
D
E
F
G
H
Type: Reference
Name: Reference 00001
rConc.: 1
Ref.:
Type: Unknown
Name:
rConc.: 1
Ref.:

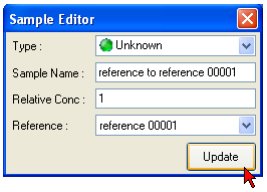
7 Select all cells that you want to reference. Typically, you select all the cells (except for the three reference cells) as in the example below.

8 Click **Editor**.

9 Enter a sample name.

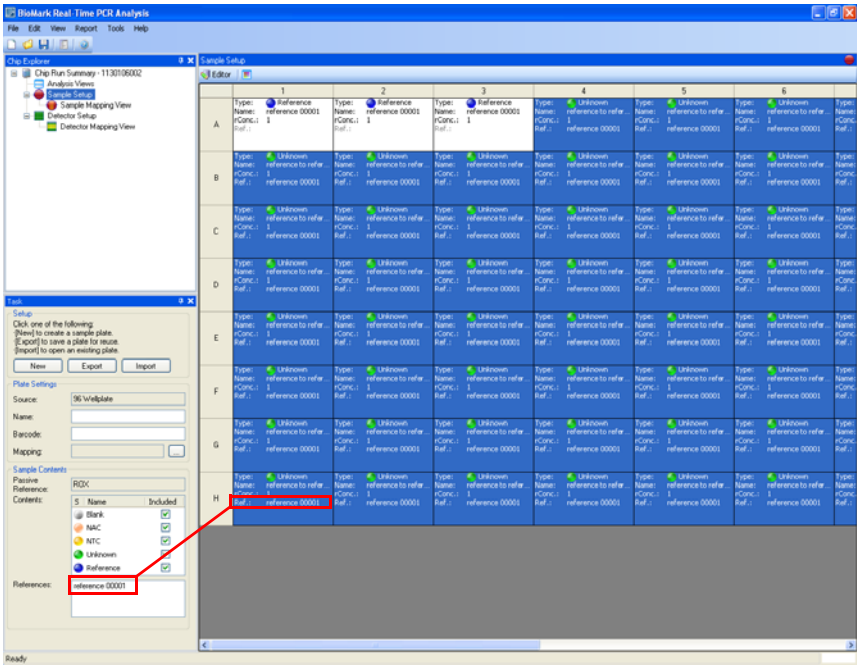
10 Select **Unknown**.

11 Select the reference you created.



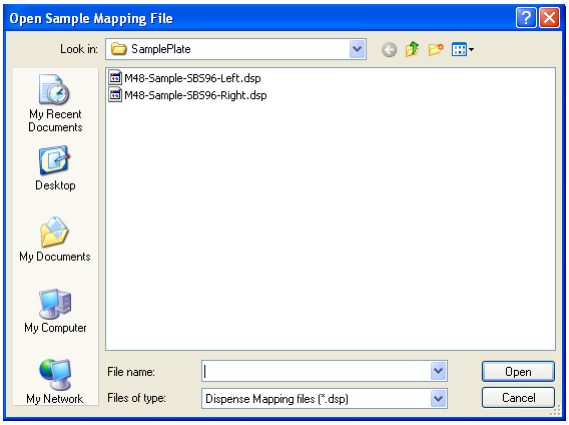
12 Click **Update**.

The changes are recorded as shown in the example below.



13 Click the **mapping** icon.

The **Open Sample Mapping File** dialog opens.

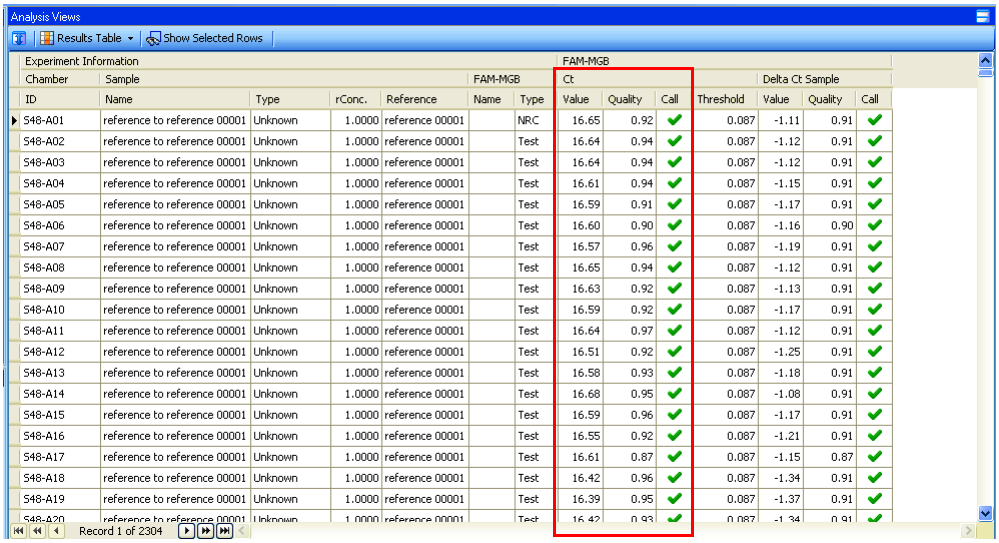


14 Double-click left or right mapping.

15 Click **Analysis Views**.

16 Click **Analyze**.

ΔC_t sample values are now available in the **Results Table** view.

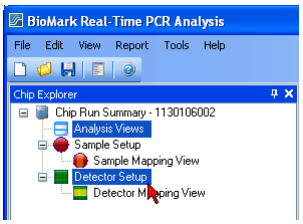

Analysis Views
Results Table
Show Selected Rows
Experiment Information
FAM-MGB
Chamber ID
Sample
Name
Type
rConc.
Reference
FAM-MGB
Name
Type
Ct
Value
Quality
Call
Threshold
Delta Ct Sample
Value
Quality
Call
S48-A01 reference to reference 00001 Unknown 1.0000 reference 00001 NRC 16.65 0.92 0.087 -1.11 0.91
S48-A02 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.64 0.94 0.087 -1.12 0.91
S48-A03 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.64 0.94 0.087 -1.12 0.91
S48-A04 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.61 0.94 0.087 -1.15 0.91
S48-A05 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.59 0.91 0.087 -1.17 0.91
S48-A06 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.60 0.90 0.087 -1.16 0.90
S48-A07 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.57 0.96 0.087 -1.19 0.91
S48-A08 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.65 0.94 0.087 -1.12 0.91
S48-A09 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.63 0.92 0.087 -1.13 0.91
S48-A10 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.59 0.92 0.087 -1.17 0.91
S48-A11 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.64 0.97 0.087 -1.12 0.91
S48-A12 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.51 0.92 0.087 -1.25 0.91
S48-A13 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.58 0.93 0.087 -1.18 0.91
S48-A14 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.68 0.95 0.087 -1.08 0.91
S48-A15 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.59 0.96 0.087 -1.17 0.91
S48-A16 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.55 0.92 0.087 -1.21 0.91
S48-A17 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.61 0.87 0.087 -1.15 0.87
S48-A18 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.42 0.96 0.087 -1.34 0.91
S48-A19 reference to reference 00001 Unknown 1.0000 reference 00001 Test 16.39 0.95 0.087 -1.37 0.91
Record 1 of 2304

Calculating Delta C_t Detector Values

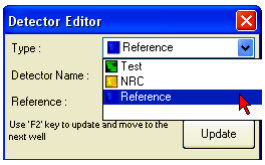
To calculate ΔC_t detector values, follow the procedure described in the previous section, "Calculating Delta Ct Sample Values" on page 105.

Steps that are specific to the procedure for calculating ΔC_t detector values are described below.

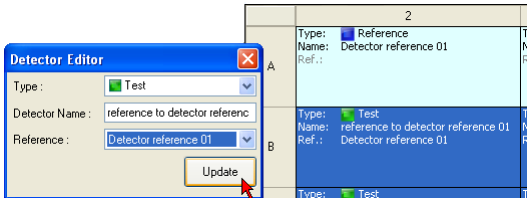
- Click **Detector Setup**.



- Click **Reference**.



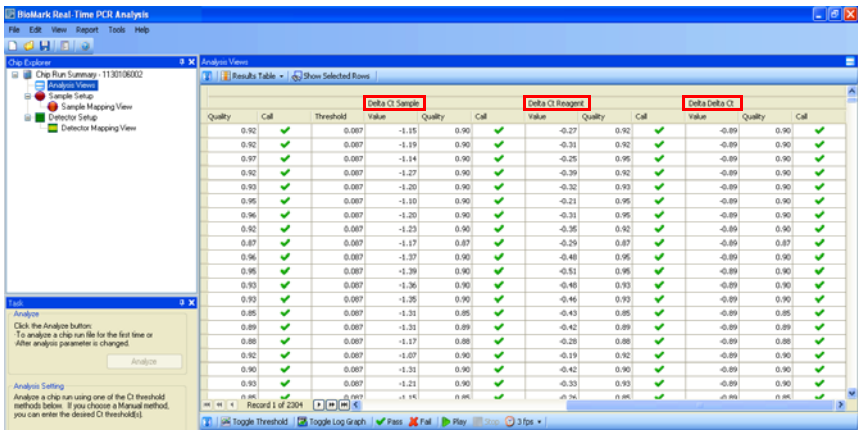
- Click **Test**.



Delta-Delta C_t Values

The $\Delta\Delta C_t$ values are available to you after the sample and the detector ΔC_t values are calculated (see "Calculating Delta Ct Detector Values" on page 109 and, "Calculating Delta Ct Sample Values" on page 105).

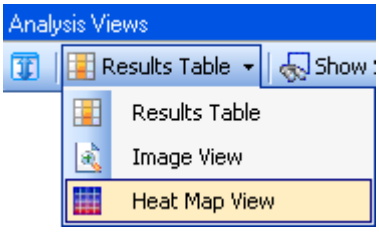
When sample and detector ΔC_t values have been calculated, click Analysis Views to see $\Delta\Delta C_t$ data:



Viewing Delta C_t Data in the Heat Map

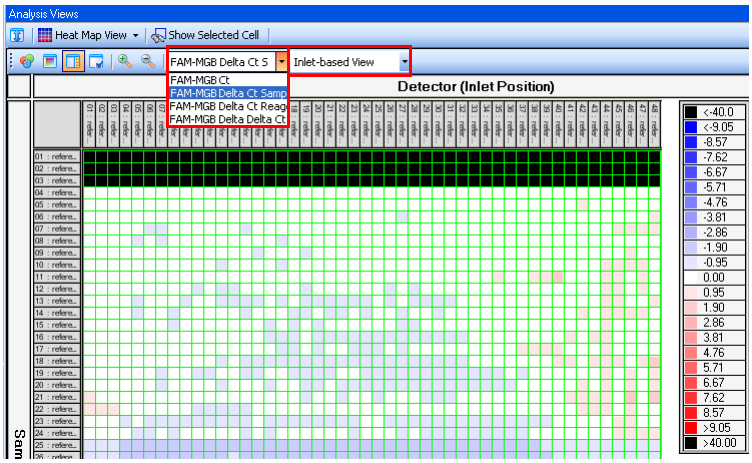
In addition to viewing ΔC_t and $\Delta\Delta C_t$ data in the Results Table, view ΔC_t data in the Heat Map.

1. Click **Analysis View**.
2. Click **Analyze**, if necessary.
3. Click **Heat Map View** from the **Results Table** menu.



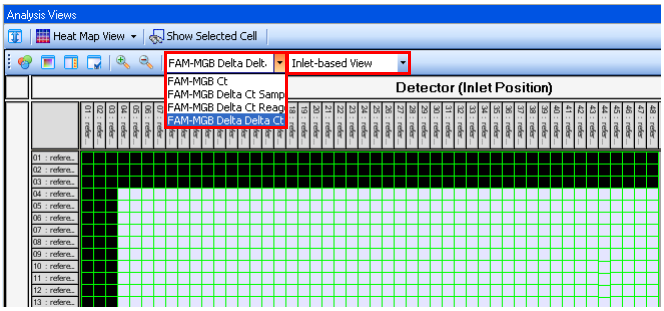
4 Select **FAM-MGB Delta C_t Sample** from the menu.

Reference cells:



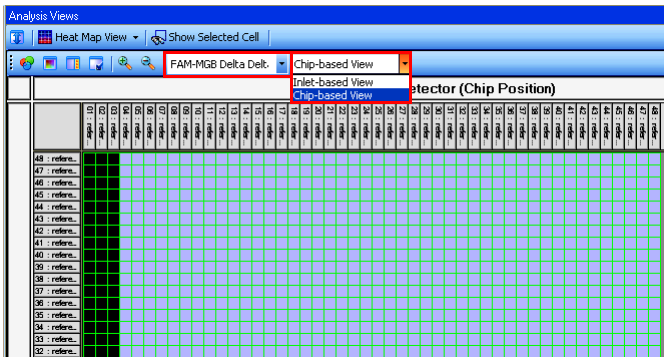
FAM-MGB Delta-Delta C_t Heat Map Data with Inlet-Based View

Reference cells:



FAM-MGB Delta-Delta C_t Heat Map Data with a Chip-Based View

Reference cells:



Congratulations, you have successfully viewed your analyzed chip run data.

Viewing Chip Run Data in the Calibration Curve View

4

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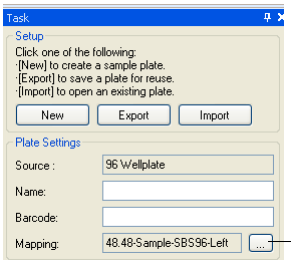
Introduction

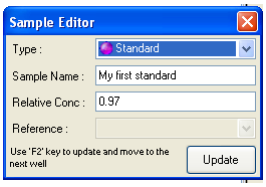
The Calibration Curve View Module (CCVM) (also known as "standard curve") is a view that allows the user to create calibration curves based on the C_t and known concentration differences of samples on the chip. After calibration curves are created, they are used to determine the approximate concentration of unknown samples on the chip. The approximate values are displayed in a table format.

For the CCVM to appear:

1. Open an unanalyzed chip run. See "Opening an Existing Chip Run" on page 29 for more information.
2. Select **Analysis Views**.
3. Click the **Analyze** button.
4. Select **Sample Setup**.
5. Click **New** to set up a new sample plate. Choose **SBS plate** or **Sample Inlet** for your container type. (For more detail, see "Setting Up a Sample Plate" on page 33.)
6. Select a Mapping option (left- or right-side maps).



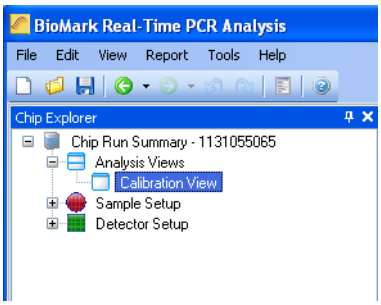
7. Use the **Editor** to annotate your sample cells. Make sure at least two wells are **Standard** type (essential for CCVM), are named, and have concentration values.



8. Set up Detector plate. For more detailed information see "Setting up a Detector (Assay) Plate" on page 44.
9. Select **Analysis Views**.
10. Click the **Analyze** button.

    The Analysis Views item now has a plus sign in front of it.

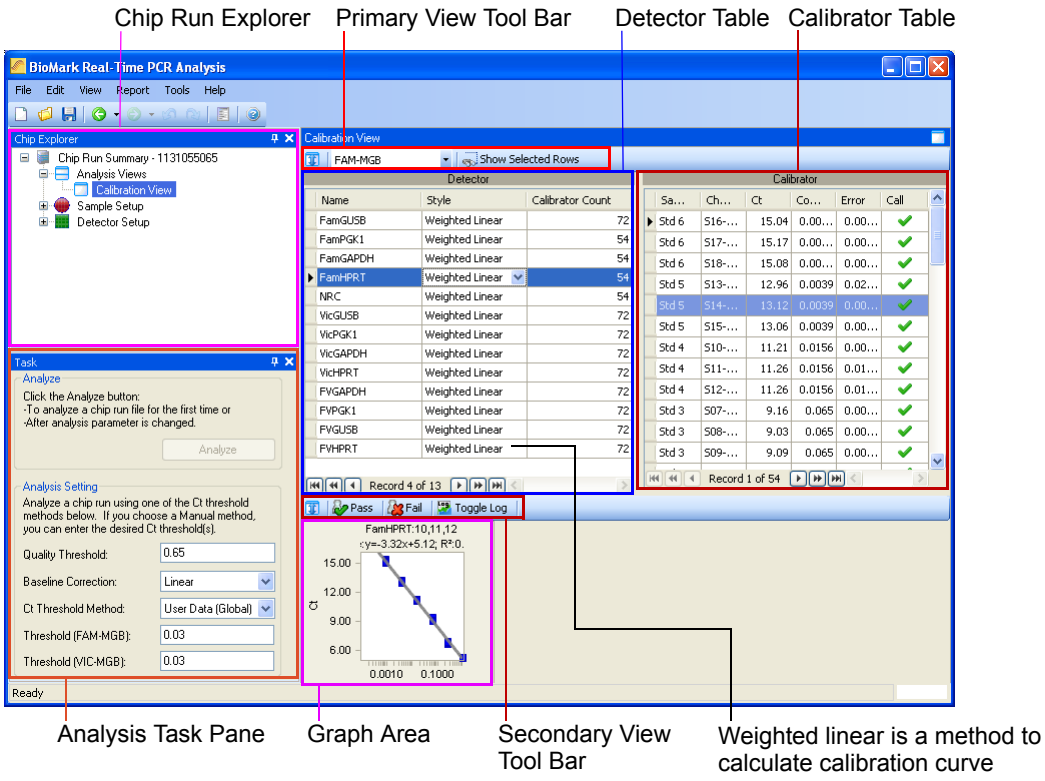
11 Expand the plus sign (+) next to **Analysis Views** and the **Calibration View** option appears on the tree.



12 Select **Calibration View** to launch the CCVM page.

CCVM Page Example

Below is the CCVM page, which consists of five individual panes and two tool bars.



Using CCVM to Determine Concentration Levels of Unknown Samples

The objective of using CCVM, is to set up a chip run with at least two standard type wells (where the concentration of DNA is known) and to predict the concentration of the unknown type samples. Four to six standard type samples are recommended. The calls of experiments that include standard samples are then plotted on the graph pane.

CCVM also allows you to modify calls associated with calibrators. This action is the same as modifying calls in the other views, but also has effect of adding or removing datapoints from the regression line (calibration curve) calculation.

When first launched, CCVM displays the contents and calibration curves of the data. These curves are created with a default fitting method (weighted linear).

1 In the Primary View Tool Bar, select a probe type, such as FAM MGB.

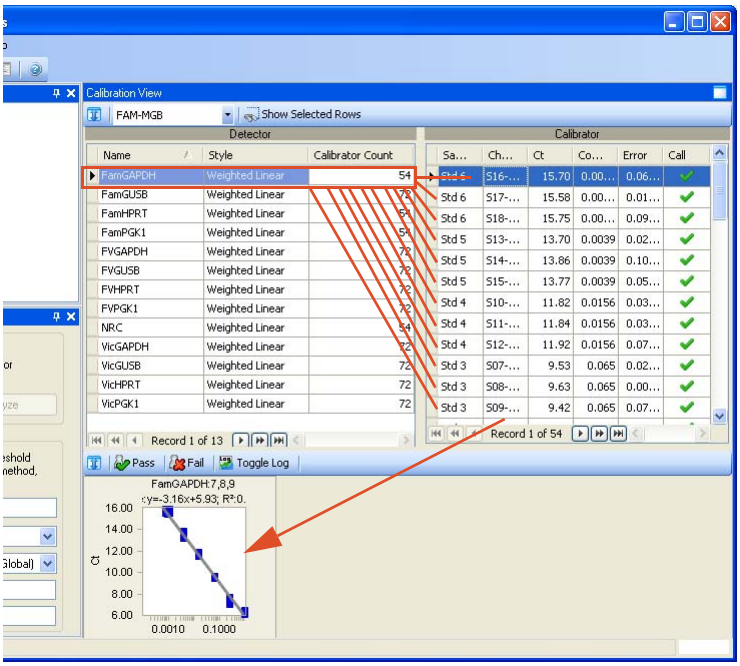
2 On the CCVM page, click on a detector in the Detector Table pane.

The Detector Table displays the attributes of a detector in three columns: Name of detector, Style of fitting method and Count of Calibrators for this row.

If detectors are named the same name, they are listed on one row in the table and the total of all calibrators are listed in the third column.

3 Adjacent to the Detector Table is a list of the calibrators applied to that detector. In the graph area below, valid detectors are plotted.

The Calibrator Table displays six attributes of a calibrator: Name, Chamber ID, C_t value, Concentration, Error, and Call.



Select a detector and its corresponding calibrators are listed on the right pane. The selected experiments are plotted on the graph area below.

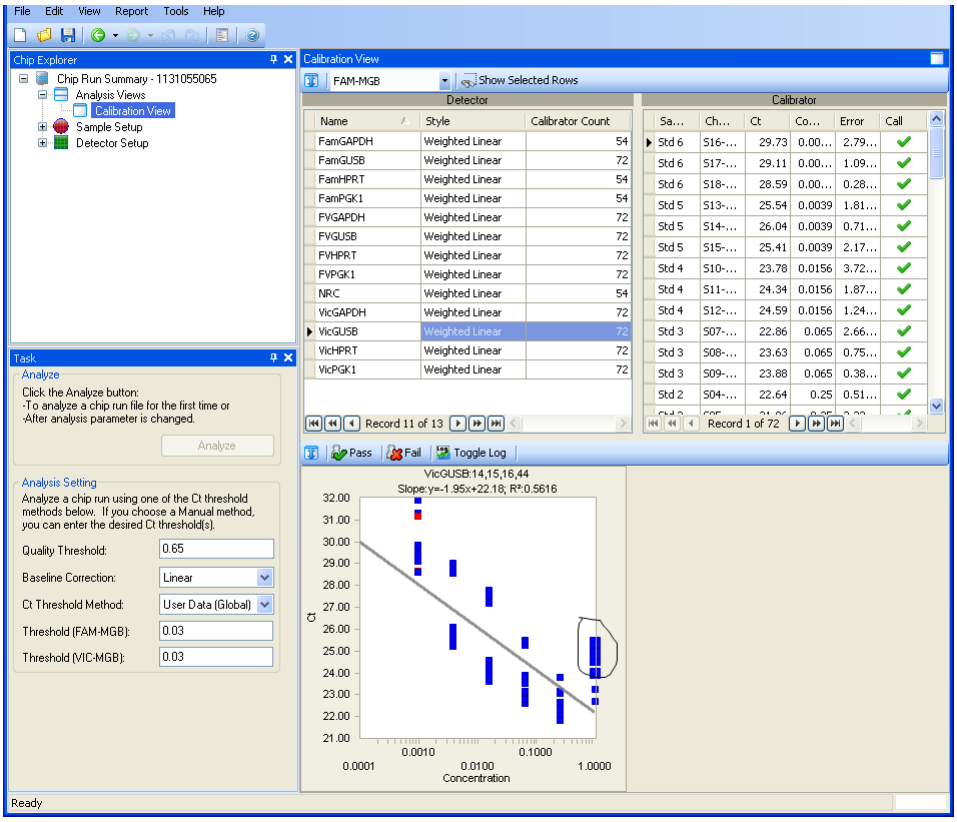
4 Select a row in the Calibrator Table and the corresponding data point in the calibration curve becomes larger. Conversely, you can lasso or click on a data point in the chart and the corresponding row in the Calibration Table is highlighted.

NOTE: You can lasso a point by pressing the left mouse button and dragging the mouse around the data point(s) to create a circle.

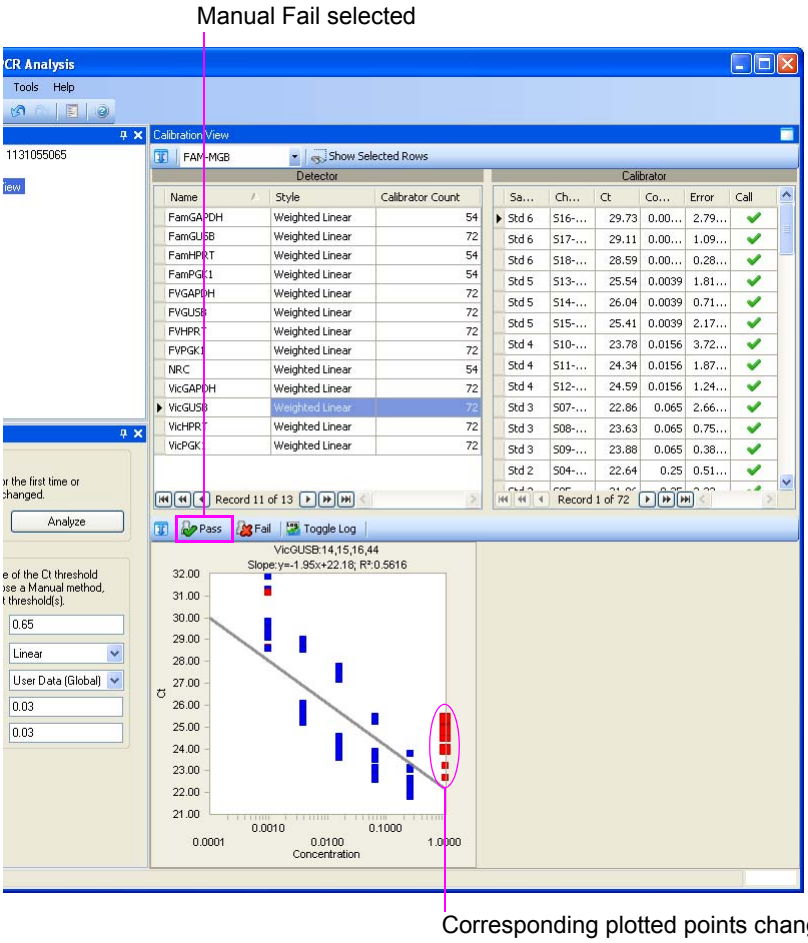
Only calibrators with valid C_t values are plotted in the calibration curve. Invalid C_t values are listed as 999. Calibrators that are auto or manual passed are plotted as blue dots. They are considered valid calibrators. Calibrators that are manually failed or passed are plotted as red dots and are considered invalid calibrators. CCVM only uses blue data points to create calibration curves. If there are no valid calibrators, no calibration curve is drawn.

5 You can modify the calls by manually changing the calibrators' calls to Pass or Fail via the secondary view tool bar.

a Select a calibrator you wish to change (you can select the row in the Calibrator Table or lasso a data point on the chart).



b Click either Pass or Fail on the secondary tool bar. Fail turns the points red. Pass turns the points blue. The corresponding calibrator in the Calibration Table changes its call accordingly and the Call column is updated.

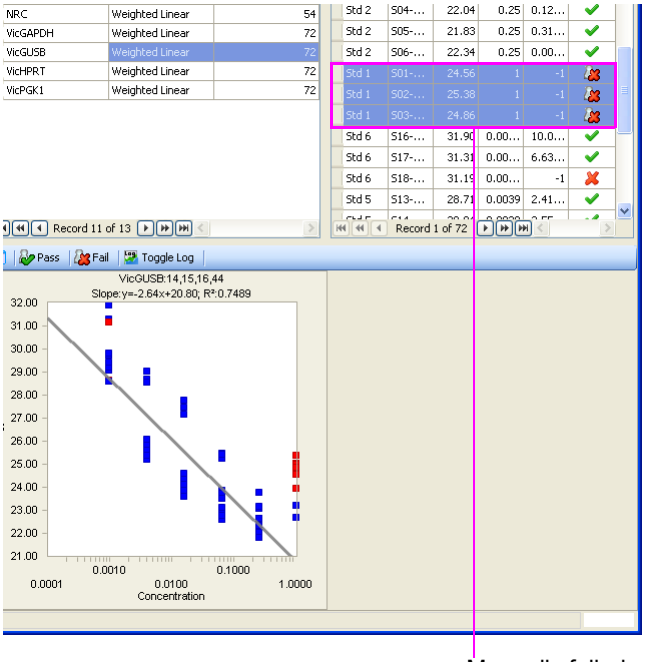


NOTE: You can use the Call Redo or Undo buttons to revert back to the original call state.

c Go to the **Analysis Views** page.

d Click the **Analyze** button to re-analyze the chip with these new parameters.

e The resulting calibration curve is slightly modified.

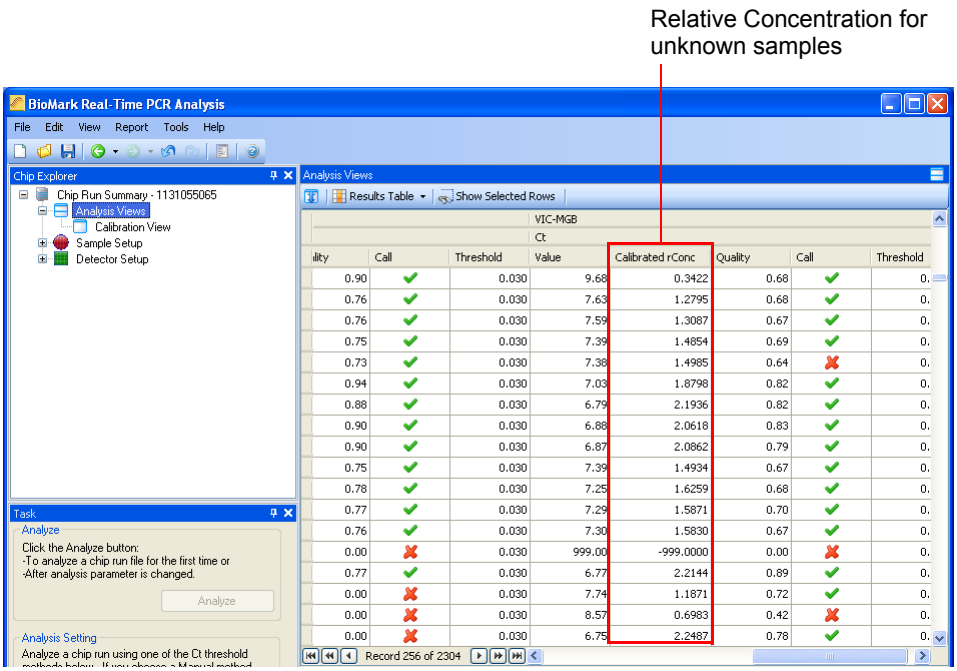


The analysis software can now use the calibration curve data to predict the approximate concentration of unknown sample types.

6 Go to the **Analysis Views** page.

7 Select the **Results Table**.

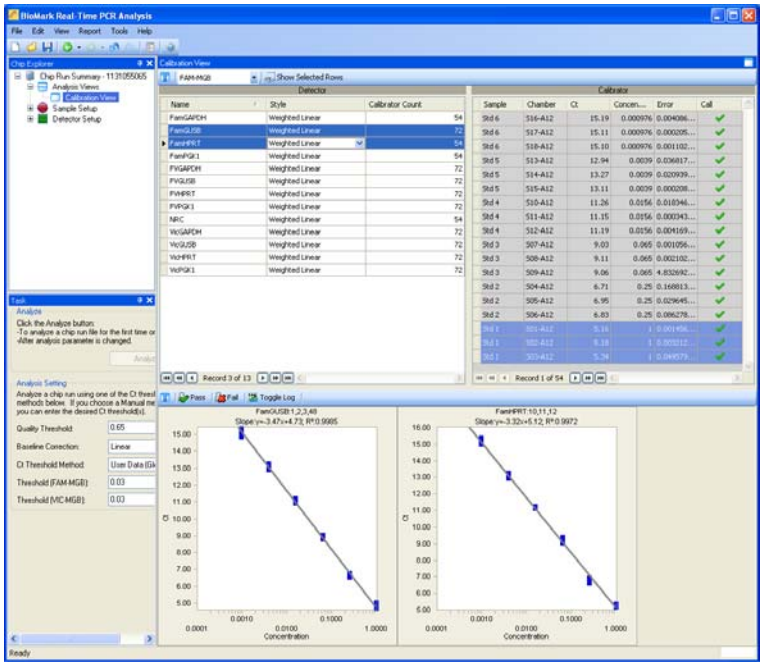
8 The approximate values are listed in the “Calibrated rConc” (Calibrated relative concentration) column.



Viewing Multiple Calibration Curves

You can also view multiple calibration curves at once on the CCVM page. To select multiple rows of assay in the Detector Table, Ctrl + left mouse click the rows of interest. Individual curves are rendered in the Graph Area.

Note, however, that the Calibrator Table behaves differently when multiple rows are selected in the Detector Table. The Calibrator Table is masked gray and the user cannot perform any actions on it. This is because the Calibrator Table was designed to only show calibrators for one detector at a time.



qPCR + Melting Curve Analysis 5

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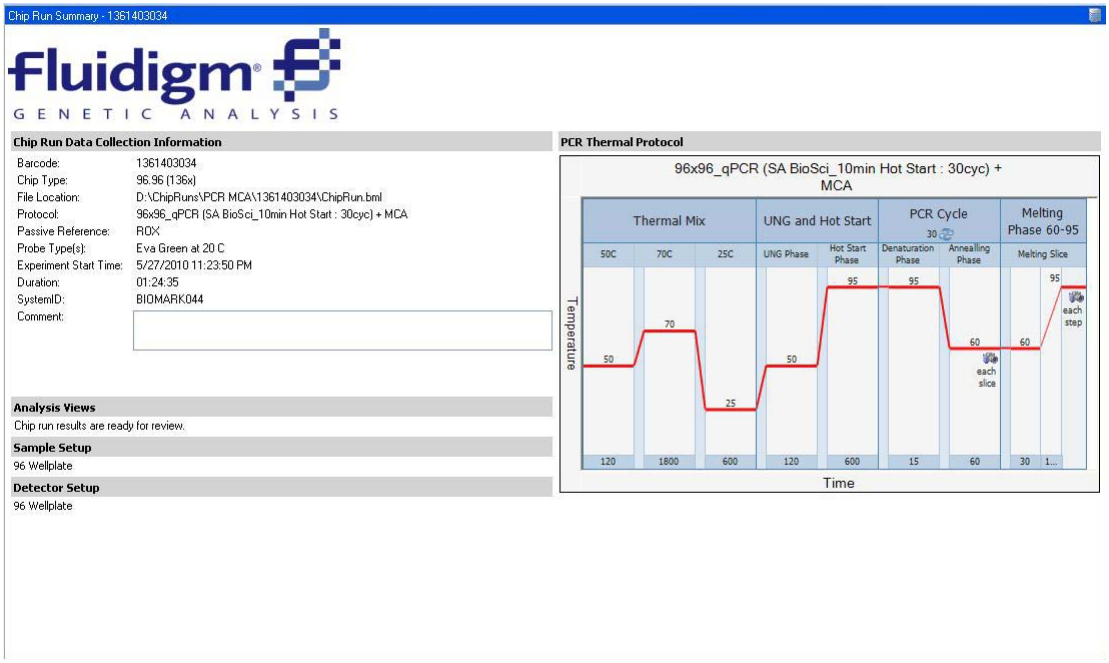
Introduction to qPCR + MCA Chip Runs

When a DNA binding dye is used for detecting PCR products, the products of the reaction can be analyzed by following the run with Melt Curve Analysis (MCA).

This chapter provides a brief overview for when you analyze a chip run that has been run with a qPCR+MCA protocol.



The protocol must have one PCR segment that is before one Melting Curve segment. This applies to any real-time chip type.

By running a Melting Curve segment after the PCR segment, th e T_m data is generated and used to validate the C_t curves of the PCR segment. The software detects up to two T_m peaks, one in a user-defined range and one outside it.

Running a Chip with a qPCR + MCA Protocol

When you run a chip with a qPCR and MCA protocol, the T_m peak detected inside the T_m detection range validates the amplification curve of the PCR cycle. If no T_m peak is detected, then any amplification that may exist is not considered valid and the quality score of the C_t is set to zero (0), making the chamber Fail.

You analyze data by clicking the **Analyze** button.

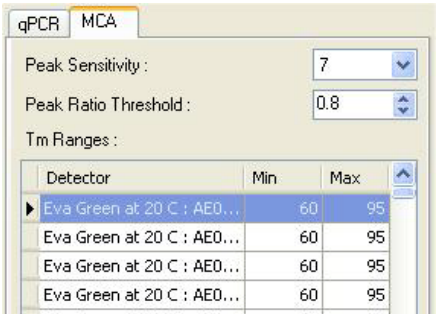
The T_m Ranges

The T_m peak detection range is set for each detector. By default, each detector range is the temperature range of the protocol.

To identify the temperature range in which you expect to see a T_m peak, select a region of the temperature range. A T_m peak in range validates the C_t curve and a T_m peak outside of range invalidates it, failing the chamber.

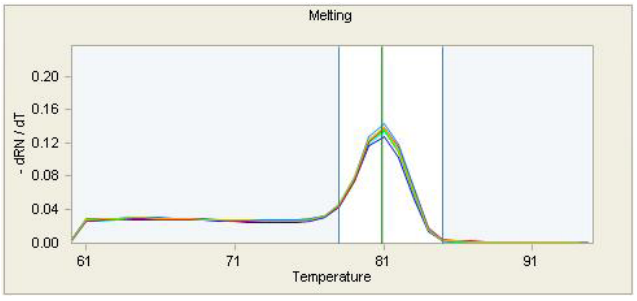
The **MCA** tab allows you change these parameters:

- **Peak Sensitivity** – adjust how sensitive the algorithm is for detecting a peak, with 1 being the least sensitive and 10 being the most sensitive.
- **Peak Ratio Threshold** – determine if a peak outside of range should cause the chamber to fail when multiple peaks are detected (one in range and one out of range)
- **T_m Ranges** – adjust the detection range for each detector.



Viewing the T_m Ranges

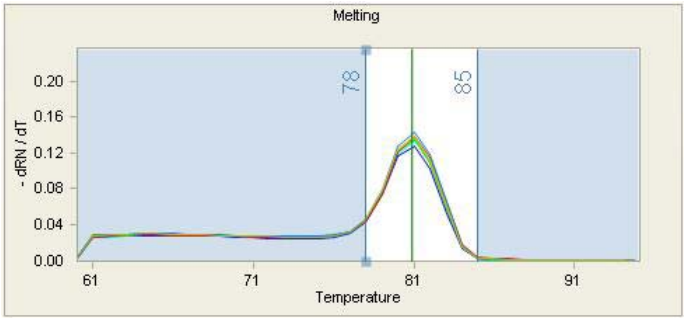
Toggle the **Threshold** button to display the T_m detection range (light blue).



Editing the Tm Ranges

Toggle the **Threshold / Edit** button to display the **MCA** tab and directly change the T_m ranges for each detector.

You can also click and drag the T_m ranges.



Working with Views

There are three views:

- Results Table
- Heat Map
- Graph

Results Table View

A T_m section appears for each probe type. The first column is the value for the T_m peak detected in range. The second column is the value for the out of range T_m peak. A value of 999 means no peak was detected.

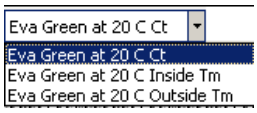
.

Tm

In Range	Out Range	Peak Ratio	Peak Call
71.39	999.00	1.00	✔
74.10	999.00	1.00	✔
74.73	999.00	1.00	✔
82.03	999.00	1.00	✔
82.20	999.00	1.00	✔

Heat Map View

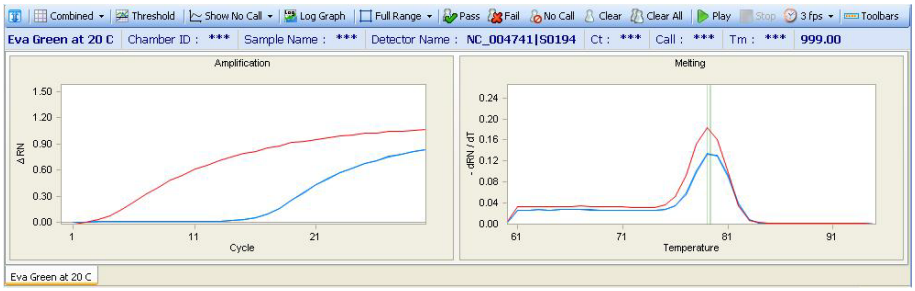
For MCA runs, there are two additional data views: Inside T_m and Outside T_m. Also, the spectrum is adjusted for T_m values.



Graph View

In this view, there are two layouts: **Combined** and **Tabbed**.

the **Combined** view shows the visible Amplification (C_t) and Melting (T_m) graphs.



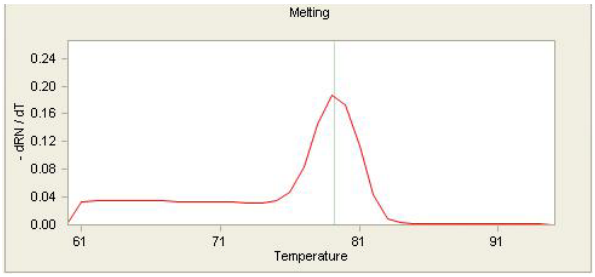
The **Tabbed** view has two tabs of graphs for each probe type defined in the chip run: **qPCR** and **MCA**.

The **qPCR** tab shows Normalized Intensity (PCR) and Amplification.

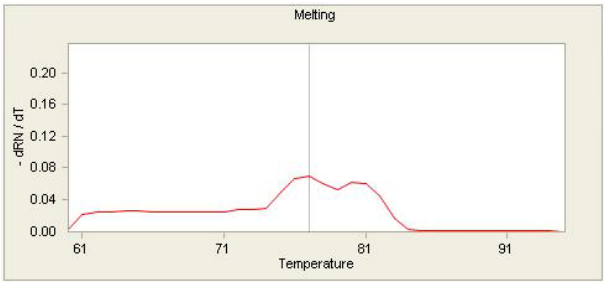
The **MCA** tab shows graphs for Normalized Intensity (MCA) and Melting.

The Melting Graph

The **Melting** graph displays the T_m curve for the selected chambers. A green vertical line represents a T_m peak inside the T_m detection range.



A black vertical line represents a T_m peak detected outside the T_m detection range.



Exporting Data

CSV Table data -- includes the T_m columns from the **Table** View.

CSV Heat Map data -- includes visible data in the heat map (C_t, Inside T_m, Outside T_m.)

Fast Gene Expression Analysis Using EvaGreen® on the BioMark™ or BioMark HD System

A

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Introduction

The use of DNA binding dyes for gene expression analysis is a lower cost alternative to the use of labeled probes. The method is sensitive and when coupled with melt curve analysis the specificity of the primers can be confirmed. For this protocol we are recommending the use of EvaGreen® dye, which has several advantages over SYBR® Green I (1, 2). This document provides a fast cycling protocol that can be used on either the BioMark™ HD with fast ramp rates (5.5ºC/s) or the BioMark with the normal ramp rate (2ºC/s). This protocol can be used with the 48.48 Dynamic Array™ integrated fluidic circuit (IFC) or the 96.96 Dynamic Array IFC.

The use of the fast ramp rate on the BioMark HD System requires the use of a PCR master mix that has been optimized for fast cycling. The fast master mix recommended for use in this protocol includes both EvaGreen® and ROX in the master mix, which makes it convenient to use. This master mix also works well on the BioMark System with the normal ramp of 2ºC/s. The total cycling time on the BioMark System will be longer than the cycling time on the BioMark HD System, but still faster than standard protocols.

Primers need to be designed to reduce the potential for primer dimer formation and to be highly specific for the target of interest. For the development of this protocol, we used DELTAgene™ Assays, a set of assays designed by the Assay Design Group at Fluidigm which avoid SNPs and are highly specific for the gene of interest.

We recommend the use of specific target amplification (STA) to increase the number of copies of target DNA. Prior to qPCR reactions the STA reaction is treated with Exonuclease I to eliminate the carryover of unincorporated primers.

References

1 SsoFast™ EvaGreen® Supermix With Low ROX product literature (http://www.bio-rad.com)

2 Mao F, Leung W-Y, and Xin X. 2007. Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. BMC Biotechnology 7:76 (doi:10.1186/1472-6750-7-76)

Required Reagents

Stored at -20°C

- Exonuclease I (New England BioLabs, PN M0293L)
- SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, PN 172-5211)

Stored at 4°C

- 2X TaqMan® PreAmp Master Mix (Applied Biosystems, PN 4391128)
- 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)

- 100 µM each Forward and Reverse Primer Stock Mixture for each assay of interest

Stored at Room Temperature

- TE Buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA) (TEKnova, PN T0224)
- PCR Certified Water (TEKnova, PN W3330)

Required Equipment

- BioMark or BioMark HD System
- IFC Controller MX (for the 48.48 Dynamic Array IFC) or HX (for the 96.96 Dynamic Array IFC)
- Standard 96-well Thermal Cycler

Software Requirements

Fluidigm® Real-Time PCR Analysis Software v.3.0.2 or higher and BioMark HD Data Collection Software v.3.1.2 or higher is required for this protocol.

Specific Target Amplification (STA)

We recommend using DELTAgene™ Assays from Fluidigm. These assays are provided as a Forward and Reverse primer mix with each primer at a concentration of 100 µM.

NOTE: If you obtain primers from another source, combine the Forward primer and Reverse primer for each assay so that the concentration of each primer is 100 µM. Proceed to step 1 below.

1 Prepare 500 nM primer mixture (10X) for Specific Target Amplification (STA):

- Pool together 1 µL aliquots of all of the 100 µM primer sets to be included in the STA reaction up to 100 assays.
- Add DNA Suspension Buffer to make the final volume 200 µL.
- The volumes used in this step can be scaled up as needed.

2 Prepare Pre-Mix and Samples for STA:

- In a DNA-free hood, prepare a Pre-Mix for the STA reaction as indicated:

Component	Volume / Reaction (µL)	Volume for 48 Reactions + 10% Overage (µL)	Volume for 96 Reactions + 10% Overage (µL)
2X TaqMan PreAmp Master Mix (Applied BioSystems)	2.5	132	264
500 nM (10X) pooled primer mixture	0.5	26.4	52.8
Water	0.75	39.6	79.2
cDNA	1.25		
Total Volume	5.0		

Table 1 STA Reaction solution

- Aliquot 3.75 µL of STA Pre-Mix for each sample.
- Remove from the DNA free hood and add 1.25 µL of cDNA to each, making a final volume of 5 µL.
- Vortex to mix the reactions and centrifuge for 10 seconds.

3 Thermal cycle the STA reactions:

- Place the STA reactions from Step 2 in a thermal cycler and cycle as indicated:

Condition	Hold	10-14 Cycles		Hold
Temperature	95ºC	95ºC	60ºC	4ºC
Time	10 minutes	15 seconds	4 minutes	∞

Table 2 Thermal cycle conditions

4 Clean up the STA amplification reaction products and dilution of final reaction products:

Exonuclease I (Exo I) Treatment Method

For best results, we recommend using a cleanup step to remove unincorporated primers. This can be done with Exonuclease I (*E. coli*).

1 Dilute the Exonuclease I to 4U/µL as shown:

Component	Per 5 µL Sample (µL)	48 Samples with Overage (µL)	96 Samples with Overage (µL)
Water	1.4	84	168
Exonuclease I Reaction Buffer	0.2	12	24
Exonuclease I at 20 units/µL	0.4	24	48
Total Volume	2.0	120	240

Table 3 Exo1 reaction solution

2 Add 2 µL of diluted Exo I at 4 U/µL to each 5 µL STA reaction, vortex, centrifuge and place in a thermal cycler.

Condition	Digest	Inactivate	Hold
Temperature	37ºC	80ºC	4ºC
Time	30 minutes	15 minutes	∞

Table 4 Thermal cycle conditions

3 Dilute the final products to an appropriate concentration for testing. The minimum amount of dilution that should be used is 5-fold but if the C_q values are consistently below 6 for some of the assays this may need to be increased to 10-fold or 20-fold. Use TE Buffer (TEKnova, PN T0224) to dilute the products as shown below:

	Volume to Add		
Volume of STA Reaction + Exonuclease I	5-fold dilution	10-fold dilution	20-fold dilution
7 µL	18 µL	43 µL	93 µL

Table 5 Dilution table

- Store diluted STA products at -20°C or use immediately for on-chip PCR.

NOTE: For larger volume STA reactions adjust the amounts of materials proportionally.

Preparing Sample Pre-Mix and Samples

1 Prepare the Sample Pre-Mix as shown below:

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Volume for 48.48 Dynamic Array IFC (µL) (60 samples)	Volume for 96.96 Dynamic Array IFC (µL) (120 samples)
Sample Pre-Mix				
2X SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, PN 172-5211)	2.5	3.0	180.0	360.0
20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738) green cap	0.25	0.3	18.0	36.0
STA and Exo I-treated sample	2.25	2.7		
Total	5.0	6.0		

Table 6 Sample Pre-Mix solution

2 Aliquot 3.3 µL of Pre-Mix for each sample and add 2.7 µL of STA and Exo I-treated sample.

3 Vortex the Sample Mix solution for a minimum of 20 seconds, and centrifuge for at least 30 seconds. Prepared reactions can be stored for short times at 4°C until the samples are ready to be loaded into the chip.

IMPORTANT: Use caution when pipetting the 20X DNA Binding Dye Sample Loading Reagent as bubbles can be introduced.

Preparing the Assay Mix

1 Dilute the 100 µM stocks of combined Forward and Reverse Primers for each assay to a final concentration of 5 µM as shown:

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Volume for 50 µL Stock ((µL)
2X Assay Loading Reagent	2.5	3.0	25
1X DNA Suspension Buffer	2.25	2.7	22.5
100 µM each mixed Forward and Reverse Primers	0.25	0.3	2.5
Total Volume	5	6	50

Table 7 Assay Mix solution

2 Vortex the Assay Mix for a minimum of 20 seconds and centrifuge for at least 30 seconds to spin down all components.

IMPORTANT: Vortex thoroughly and centrifuge all sample and assay solutions before pipetting into the chip inlets. Failure to do so may result in a decrease in data quality.

NOTE: The final concentration of each primer is 5 µM in the inlet and 500 nM in the final reaction.

Priming and Loading the Dynamic Array IFC

CAUTION! Due to different accumulator volumes, use the appropriate control syringe for your chip type: **300 µL** for the 48.48 Dynamic Array IFC or **150 µL** for the 96.96 Dynamic Array IFC.

1 Inject control line fluid into each accumulator on the chip.

2 Remove and discard the blue protective film from the bottom of the chip.

3 Place the chip into the IFC controller MX for the 48.48 Dynamic Array IFC or the IFC Controller HX for the 96.96 Dynamic Array IFC, then run the **Prime (113x)** script for the 48.48 Dynamic Array IFC or the **Prime (136x)** script for the 96.96 Dynamic Array IFC).

4 When the script has finished, press **Eject** to remove the primed chip from the IFC Controller.

CAUTION! While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets.

5 Pipette 5 µL of each assay and 5 µL of each sample into their respective inlets on the chip.

6 Return the chip to the IFC Controller.

7 Using the IFC controller software, run the **Load Mix (113x)** script for the 48.48 Dynamic Array IFC) or **Load Mix (136x)** script for the 96.96 Dynamic Array IFC to load the samples and assays into the chip.

8 When the Load Mix script has finished, remove the loaded chip from the IFC Controller.

You are now ready for your chip run.

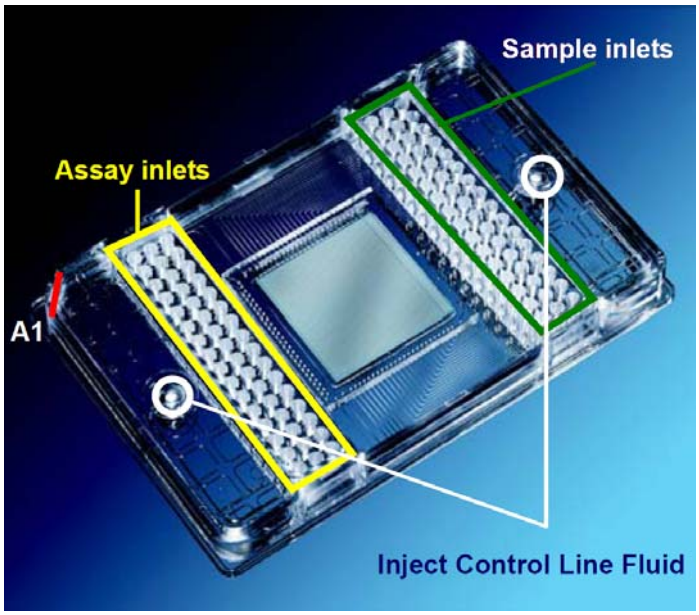

Figure 1 48.48 Dynamic Array IFC sample and assay inlets

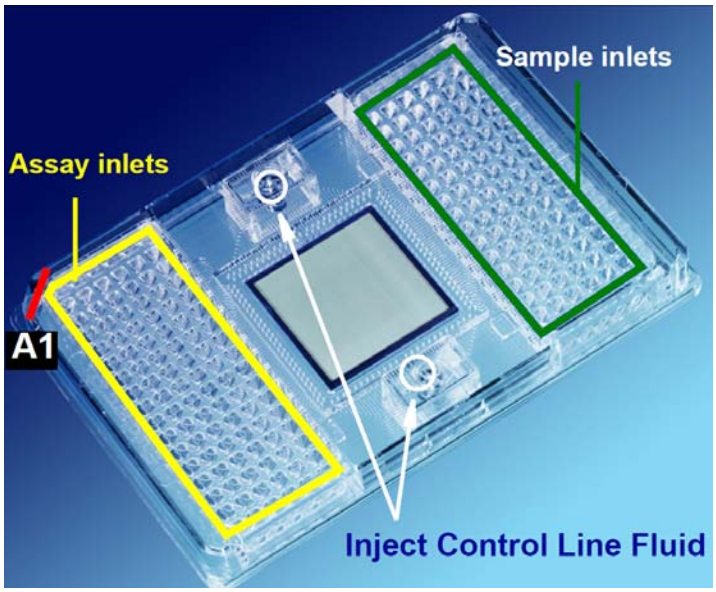

Figure 2 96.96 Dynamic Array IFC sample and assay inlets

Using the Data Collection Software

1. Double-click the Data Collection Software icon on the desktop to launch the software.
2. Click **Start a New Run**.
3. Check the status bar to verify that the camera has a green light to indicate that it is ready.
4. Remove the blue tape from the back of the chip if this was not done previously. Place the chip into the reader.
5. Click **Load**.
6. Verify chip barcode and chip type.
7. Choose project settings (if applicable).
8. Click **Next**.
9. Chip Run file:
   - **a** Select **New**.
   - **b** Browse to a file location for data storage.
   - **c** Click **Next**.
10. Application, Reference, Probes:
    - **a** Select Application Type—**Gene Expression**.
    - **b** Select Passive Reference—**ROX**.
    - **c** Select Probe—**Single probe**.
    - **d** Select Probe type—**EvaGreen**.

e Click **Next**.

11 Click **Browse** to find the thermal cycling protocol files:

For BioMark HD:

- GE Fast 48x48 PCR+Melt v2.pcl
- GE Fast 96x96 PCR+Melt v2.pcl

For BioMark:

- GE 48x48 PCR+Melt v2.pcl
- GE 96x96 PCR+Melt v2.pcl

12 Confirm **Auto Exposure** is selected.

13 Click **Next**.

14 Verify the chip run information.

15 Click **Start Run**.

The cycling parameters are given for the two different chip types:

	Segment	Type	Temperature (°C)	Duration (seconds)	BioMark HD Ramp Rate (°C/s)	BioMark Ramp Rate (°C/s)
96x96 chip	1	Thermal Mix	70	2400	5.5	2
			60	30	5.5	2
	2	Hot Start	95	60	5.5	2
	3	PCR (30 Cycles)	96	5	5.5	2
			60	20	5.5	2
	4	Melting Curve	60	3	1	1
			60-95		1°C/3 s	1ºC/3 s
48x48 chip	1	Hot Start	95	60	5.5	2
	2	PCR (30 Cycles)	96	5	5.5	2
			60	20	5.5	2
	3	Melting Curve	60	3	1	1
			60-95		1°C/3 s	1ºC/3 s

Table 8 Thermal cycle parameters

Using the Real-Time PCR Analysis Parameters

1. Double-click the Real-Time PCR Analysis software icon on the desktop to launch the software.
2. Click **Open Chip Run**.
3. Double-click a ChipRun.bml file to open it in the software.
4. Enter detector and sample information.
5. Select **Analysis Views**. We recommend using the **AutoGlobal** method to set the threshold.
6. We recommend using **Linear Derivative** as the baseline correction method. For more information about baseline correction methods, contact Fluidigm Technical Support.
7. Always compare the T_m of the intended products to a positive control sample.
8. Click **Analyze**.

NOTE: For more information about melting curve analysis, see the Fluidigm Real-Time PCR Analysis Software v 3.0 User Guide (PN 68000088).

Two-Step Single-Cell Gene Expression Using EvaGreen® Supermix on the BioMark™ or BioMark HD System

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Introduction

This protocol includes a separate reverse transcription step and a specific target amplification (STA) step, hence its "two-step" title. The following protocol enables the use of a DNA binding dye for quantitative PCR gene expression. DNA binding dyes offer flexibility at a very low upfront cost relative to probe-based assays, and can be used for a variety of applications. This specific protocol has been tested for gene expression targeting 1 and 10 cells on the BioMark™ and BioMark HD System and should serve only as a guideline for any customers interested in qPCR dye experiments. We recommend examining melting curve (T_m) and C_q for all assays alongside a positive control sample.

The protocol involves performing Specific Target Amplification (STA), which enriches samples for loci of interest. STA retains relative abundance between loci and permits quantitative C_q information to be derived. See Devonshire *et al.* BMC Genomics 2011, 12.118 for more information on STA (preamplification). Quantitative PCR is then performed in the presence of a DNA binding dye, known as EvaGreen® dye. Quantitative PCR thermal cycling protocols are immediately followed by acquisition of a melting curve (T_m) to allow assessment of reaction quality. See Mao *et al., BMC Biotechnology* 2007, 7:76, for further information on the physicochemical properties of EvaGreen dye. See Devonshire *et al.*, BMC Genomics 2011, 12.118 for more information on STA (preamplification).

This two-step protocol is optimized for both the BioMark and BioMark HD systems. It includes a fast master mix and a relatively short qPCR protocol. We have validated a supermix that has EvaGreen® and ROX already incorporated. The protocol also uses a 2-step VILO™ cDNA synthesis kit.

Required Reagents

- SuperScript® VILO™cDNA Synthesis Kit (Invitrogen, PN 11754-250)
- SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, PN 172-5211)
- SUPERase-In™ RNase Inhibitor (Ambion, PN AM2696)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)
- 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738)
- DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)
- Exonuclease I (New England BioLabs, PN M0293S or M0293L)
- 0.5M EDTA, pH 8.0 (Invitrogen, PN Am9260G)
- T4 Gene 32 Protein (New England BioLabs, PN M0300S or M0300L)
- TaqMan® PreAmp Master Mix, (Applied Biosystems, PN 4391128)
- NP-40 Detergent Surfact-Amps Solution (Fisher Scientific, PN PI-28324, or Thermo Scientific, PN 28324)
- Nuclease-free Water (Teknova, PN W3330)

Required Equipment

- BioMark or BioMark HD System
- IFC Controller MX (for the 48.48 Dynamic Array IFC) or IFC Controller HX (for the 96.96 Dynamic Array IFC)
- Standard 96-well thermal cycler
- 96-well plates that are compatible with the FACS instrument (if FACS sorting) and thermal cycler
- Adhesive plate seals (Applied Biosystems, PN 4311971)

Software Requirements

Fluidigm Real-Time PCR Analysis Software v.3.1.3 or higher and Fluidigm Data Collection software v.3.1.2 or higher is recommended for this protocol. For earlier versions, contact Technical Support. Call 1-866-358-4354 (within U.S.) or 1-650-266-6100 (outside U.S.), or email techsupport@fluidigm.com.

Preparing the Reverse Transcription (RT) Reaction Assembly

1 For each well of a 96-well plate that will be used for sorting, prepare RT mix solution.

Component	Volume (µL)	48 Samples with Overage (µL)	96 Samples with Overage (µL)
5X VILO™ Reaction Mix	1.2	72.0	144.0
20U/µL SUPERase-In™	0.3	18.0	36.0
10% NP40	0.25	15.0	30.0
Nuclease-free Water	3.25	195.0	390.0
Total	**5.0**	**300.0**	**600.0**

Table 1 RT Mix Solution 1

2 Pipette 5 µL of RT Mix Solution 1 into each well to be used of the 96-well PCR plate.

3 Sort individual cells or sort up to 10 cells directly into the same plate containing RT Mix Solution 1.

NOTE: Sort cells into the same 96-well plate that will be used for thermal cycling.

4 Seal the plate and vortex thoroughly for 15 seconds.

5 Pre-chill centrifuge to 4ºC.
6 Centrifuge plate briefly at 4ºC.
7 Immediately freeze the plate on dry ice.
8 Store plate at -80ºC or thaw plate to use immediately.

Denaturation of RNA

1 When you are ready to perform RT cycling:
   a Thaw samples on ice.
   b Use a pre-chilled centrifuge maintained at 4ºC to spin the plate briefly.
   c Preheat thermal cycler to 65ºC.
   d Transfer the samples to the thermal cycler.

Condition	Denature
Temperature	65 ºC
Time	90 seconds

2 Snap chill the plate on ice immediately for 5 minutes and centrifuge briefly at 4 ºC.
3 Prepare enough RT Mix Solution 2 for all sorted wells, according to the table below.

Component	Volume (µL)	48 Samples with Overage (µL)	96 Samples with Overage (µL)
10X SuperScript® Enzyme Mix	0.15	9.0	18.0
T4 Gene 32 Protein	0.12	7.2	14.4
Nuclease-free Water	0.73	43.8	87.6
Total	**1.00**	**60.0**	**120.0**

Table 2 RT Mix Solution 2

4 Aliquot 1 µL into each of the wells and centrifuge briefly at 4 ºC.

RT Cycling

Follow the thermal cycling conditions below on a standard thermal cycler:

Condition	Reverse Transcription					Hold
Temperature	25 ºC	50 ºC	55 ºC	60 ºC	70 ºC	4 ºC
Time	5 minutes	30 minutes	25 minutes	5 minutes	10 minutes	infinity

1 Centrifuge and store the first strand cDNA samples at -20ºC or proceed directly to PCR.

Preparing 10X STA Primer Mix

1 We recommend using DELTAgene™ Assays from Fluidigm. These assays come as a Forward and Reverse primer mix with each primer at a concentration of 100 µM.

NOTE: If you obtain primers from another source, combine the Forward primer and Reverse primer for each assay so that the concentration of each primer is 100 µM. Proceed to step 2.

2 In a DNA-free hood, combine equal volumes of each 100 µM primer pair.

3 Dilute using 1X DNA Suspension Buffer so that each primer is at a final concentration of 500 nM. This mix represents a 10X concentration of pooled STA Primer Mix.

4 Vortex for 20 seconds and centrifuge for 30 seconds to spin down all components.

5 Store 10X STA Primer Mix at 4ºC for repeated usage up to six months or store at -20ºC for long-term storage.

48 Primer Pairs (EXAMPLE)	Volume (µL)
1 µL each primer pair (100 µM each)	1 µL (x 48 = 48 µL)
1X DNA Suspension Buffer	152 µL
Total	**200**

Table 3 Preparation of 500 nM (10X) pooled STA Primer Mix

Preparing STA Reaction Mix

1 For each well of a 96-well PCR plate that was used for sorting, prepare the following mix.

Component	Per 9 µL Sample (µL)	48 Samples with Overage (µL)	96 Samples with Overage (µL)
TaqMan® PreAmp Master Mix (Invitrogen, PN 4391128)	7.5	390.0	780.0
10X STA Primer Mix (500 nM)	1.5	78.0	156.0
0.5M EDTA, pH 8.0 (Invitrogen, PN AM9260G)	0.075	3.90	7.80
Total Volume	**9.0**	**471.9**	**943.8**

Table 4 STA Reaction Mix

2 Aliquot 9 µL of the STA reaction mix to each of the first strand cDNA samples.

STA Cycling

1 Follow the thermal cycling conditions below on a standard thermal cycler.

Condition	Enzyme Activation	20 Cycles (1-10 cells)		Hold
		Denaturation	Annealing/Extension	
Temperature	95 ºC	96 ºC	60 ºC	4 ºC
Time	10 minutes	5 seconds	4 minutes	infinity

Exonuclease I (Exo I) Treatment Method

For best results, we recommend using a cleanup step to remove unincorporated primers. This can be done with Exonuclease I (*E.coli*).

1 Just before use, dilute the Exonuclease I to 4U/µL as shown:

Component	Per 15 µL Sample (µL)	48 Samples with Overage (µL)	96 Samples with Overage (µL)
Water	4.2	252.0	504.0
Exonuclease I Reaction Buffer (10X)	0.6	36.0	72.0
Exonuclease I at 20 units/µL	1.2	72.0	144.0
Total Volume	6.0	360.0	720.0

Table 5 Exo 1 Reaction Solution

2 Add 6 µL of diluted Exo I at 4 U/µL to each 15 µL STA reaction, vortex, centrifuge and place in a thermal cycler.

Condition	Digest	Inactivate	Hold
Temperature	37ºC	80ºC	4ºC
Time	30 minutes	15 minutes	infinity

3 Dilute the final products to an appropriate concentration for testing. The minimum amount of dilution that should be used is 5-fold but if the C_t (also known as C_q) values are consistently below 6 for some of the assays this may need to be increased to 10-fold or 20-fold. Use low EDTA TE or DNA Suspension Buffer (TEKnova, PN T0221) to dilute the products as shown below:

Table 1: Dilution Table

	Volume to Add		
Volume of STA Reaction + Exonuclease I	**5-fold dilution**	**10-fold dilution**	**20-fold dilution**
21.0 µL	54.0 µL	129.0 µL	279.0 µL

Table 6 Dilution Table

4 Store diluted STA products at -20 °C or use immediately for on-chip PCR.

NOTE: For larger volume STA reactions adjust the amounts of materials proportionally.

Preparing the Sample Pre-Mix and Samples

We recommend calculating overages when preparing the Sample Pre-Mix solution. The volumes in the table below apply to a Fluidigm 48.48 Dynamic Array IFC and a Fluidigm 96.96 Dynamic Array IFC.

1 Combine the following to make the Sample Pre-Mix solution:

Table 2: Sample Pre-Mix solutions

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Volume for 48.48 Dynamic Array IFC (µL) (60 samples)	Volume for 96.96 Dynamic Array IFC (µL) (120 samples)
2X Sso Fast EvaGreen Supermix With Low ROX (Bio-Rad Laboratories, PN 172-5211)	2.5	3.0	180.0	360.0
20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738) green cap	0.25	0.3	18.0	36.0
STA and Exo I-treated sample	2.25	2.7		
Total	**5**	**6**		

IMPORTANT: Use caution when pipetting the Fluidigm 20X DNA Binding Dye Sample Loading Reagent as bubbles can be introduced.

2 In a 96-well plate, combine 3.3 µL of Sample Pre-Mix with 2.7 µL of STA and Exo I-treated sample to make a final volume of 6 µL Sample Mix solution.

3 Vortex the Sample Mix solution for a minimum of 20 seconds, and centrifuge for at least 30 seconds.

Prepared reactions can be stored at 4 ºC overnight.

Preparing the 5 µM (10X) Assay Mix

The same preparation of primers can be used for the Fluidigm 48.48 Dynamic Array IFC and the Fluidigm 96.96 Dynamic Array IFC. Prepare primers as shown below.

1 Combine the following:

Table 3: Assay Mix solutions

Component	Volume for per Inlet (µL)	Volume for per Inlet with Overage (µL)	Volume for 50 µL Stock
2X Assay Loading Reagent (Fluidigm, PN 85000736) yellow cap	2.5	3.0	25.0
1X DNA Suspension Buffer (Teknova, PN T0221)	2.25	2.7	22.5
100 µM each of Forward and Reverse Primer Mix	0.25	0.3	2.5
Total	**5.0**	**6.0**	**50.0**

2 Vortex the Assay Mix for a minimum of 20 seconds, and centrifuge for at least 30 seconds to spin down all components.

IMPORTANT: Vortex thoroughly and centrifuge all sample and assay solutions before pipetting into the chip inlets. Failure to do so may result in a decrease in data quality.

NOTE: The final concentration of each primer is 5 µM in the inlet and 500 nM in the final reaction.

Priming the Chip and Loading Assay and Samples

CAUTION! Due to different accumulator volumes, use the appropriate control syringe for your chip type: **300 µL** (for the 48.48 Dynamic Array IFC) or **150 µL** (for the 96.96 Dynamic Array IFC).

1. Inject control line fluid into each accumulator on the chip (see Figure 1 for the 48.48 Dynamic Array IFC or Figure 2 for the 96.96 Dynamic Array IFC).
2. Remove and discard the blue protective film from the bottom of the chip.
3. Place the chip into the IFC Controller MX (for the 48.48 Dynamic Array IFC) or the IFC Controller HX (for the 96.96 Dynamic Array IFC), then run the **Prime (113x)** script (for the 48.48 Dynamic Array IFC) or the **Prime (136x)** script (for the 96.96 Dynamic Array IFC).
4. When the **Prime** script has finished, press **Eject** to remove the primed chip from the IFC Controller.

CAUTION! While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets.

5. Pipette 5 µL of each assay and 5 µL of each sample into their respective inlets on the chip.
6. Return the chip to the IFC Controller.
7. Using the IFC Controller software, run the **Load Mix (113x)** script (for the 48.48 Dynamic Array IFC) or **Load Mix (136x)** script (for the 96.96 Dynamic Array IFC) to load the samples and assays into the chip.
8. When the **Load Mix** script has finished, remove the loaded chip from the IFC Controller.
9. Remove any dust particles or debris from the chip surface using scotch tape.

   You are now ready for your chip run.

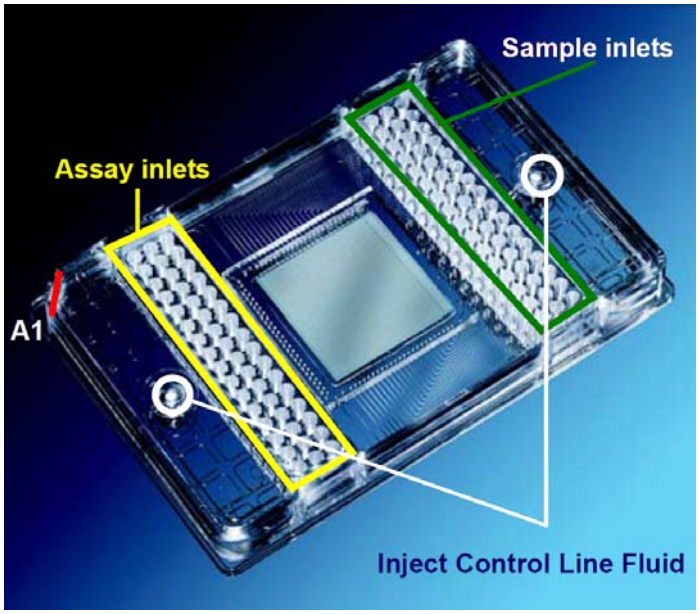

Figure 1 48.48 Dynamic Array IFC sample and assay inlets

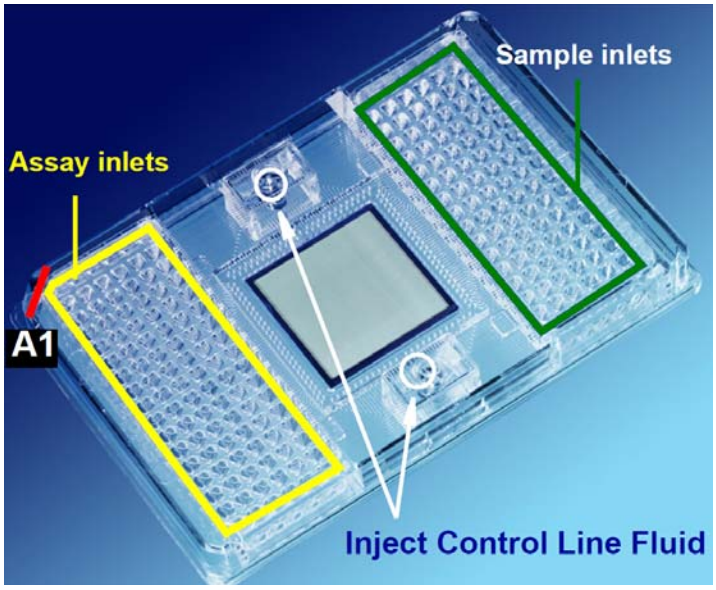

Figure 2 Figure 2. 96.96 Dynamic Array IFC sample and assay inlets

Using the Data Collection Software

1. Double-click the Data Collection Software icon on the desktop to launch the software.
2. Click **Start a New Run**.
3. Check the status bar to verify that the camera has a green light to indicate that it is ready.
4. Remove the blue tape from the back of the chip if this was not done previously. Place the chip into the reader.
5. Click **Load**.
6. Verify chip barcode and chip type.
7. Choose project settings (if applicable).
8. Click **Next**.
9. Chip Run file:
   a. Select **New**.
   b. Browse to a file location for data storage.
   c. Click **Next**.
10. Application, Reference, Probes:
    a. Select Application Type—**Gene Expression**.
    b. Select Passive Reference—**ROX**.
    c. Select Probe—**Single probe**.
    d. Select Probe type—**EvaGreen**.
    e. Click **Next**.
11. Click **Browse** to find the thermal cycling protocol files:
    For BioMark HD:
    - GE Fast 48x48 PCR+Melt v2.pcl
    - GE Fast 96x96 PCR+Melt v2.pcl

    For BioMark:
    - GE 48x48 PCR+Melt v2.pcl
    - GE 96x96 PCR+Melt v2.pcl
12. Confirm **Auto Exposure** is selected.
13. Click **Next**.
14. Verify the chip run information.

15 Click **Start Run**.

The cycling parameters are given for the two different chip types:

Segment	Type	Temperature (°C)	Duration (seconds)	BioMark HD Ramp Rate (°C/s)	BioMark Ramp Rate (°C/s)
1	Thermal Mix	70	2400	5.5	2
		60	30	5.5	2
2	Hot Start	95	60	5.5	2
3	PCR (30 Cycles)	96	5	5.5	2
		60	20	5.5	2
4	Melting Curve	60	3	1	1
		60-95		1 °C/3 s	1 ºC/3 s
1	Hot Start	95	60	5.5	2
2	PCR (30 Cycles)	96	5	5.5	2
		60	20	5.5	2
3	Melting Curve	60	3	1	1
		60-95		1 °C/3 s	1 ºC/3 s

Using the Real-Time PCR Analysis Parameters

1. Double-click the Real-Time PCR Analysis software icon on the desktop to launch the software.
2. Click **Open Chip Run**.
3. Double-click a ChipRun.bml file to open it in the software.
4. Enter detector and sample information.
5. Select **Analysis Views**. We recommend using the **AutoGlobal** method to set the threshold.
6. We recommend using **Linear Derivative** as the baseline correction method. For more information about baseline correction methods, contact Fluidigm Technical Support.
7. Always compare the T_m of the intended products to a positive control sample.

8 Click **Analyze**.

NOTE: For more information about melting curve analysis, see the Fluidigm Real-Time PCR Analysis Software v 3.0 User Guide (PN 68000088).

Fast Gene Expression Analysis Using TaqMan Gene Expression Assays

C

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Introduction

This protocol is intended to be used for fast gene expression analysis on the BioMark™ HD System using TaqMan® Gene Expression Assays. The protocol is suitable for use with either 48.48 or 96.96 Dynamic Array™ IFCs and appropriate cycling protocols are provided for each chip type. This protocol requires a BioMark HD System, which includes a thermal cycler with fast cycling capabilities. The protocol also requires the use of a master mix that does not require a long hot start and that works well with the shortened cycling times. Three master mixes that we have found to work well are Quanta PerfeCTa™ qPCR Fast Mix from Quanta Biosciences (also available from VWR), TaqMan Fast Universal Master Mix from Applied Biosystems, and TaqMan GTXpress Master Mix from Applied Biosystems. In preliminary testing, the new TaqMan Fast Advanced Master Mix from Applied Biosystems also appears to be suitable for use with this protocol. Although the four master mixes above are recommended because they work well for fast gene expression analysis, they also can be used with standard cycling conditions.

This protocol was thoroughly tested on a wide variety of assays and good results can be expected from the majority of assays. For especially difficult assays, the cycling conditions can be modified.

Required Reagents

- 2X TaqMan PreAmp Master Mix (Applied Biosystems, PN 4391128)
- 20X TaqMan Gene Expression Assays (Applied Biosystems)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)
- 2X Master Mix for Fast Cycling:
  - Quanta PerfeCTa® qPCR Fast Mix®, low ROX™ (Quanta Biosciences, PN 95078-012 or VWR, PN 101419-220) or
  - TaqMan Fast Universal PCR Master Mix (Applied Biosystems, PN 4352042) or
  - TaqMan GTXpress Master Mix (Applied Biosystems, PN 4401892) or
  - TaqMan Fast Advanced Master Mix (Applied Biosystems, PN 4444557)
- 20X GE Sample Loading Reagent (Fluidigm, PN 85000746)

Required Equipment

- Standard 96-well Thermal Cycler
- IFC Controller MX (for the 48.48 Dynamic Array IFC) or HX (for the 96.96 Dynamic Array IFC)
- BioMark HD System

Required Software

Fluidigm® Real-Time PCR Analysis Software v.3.0.2 or higher and BioMark HD Data Collection Software v.3.0.2 or higher is required for this advanced development protocol.

Specific Target Amplification (STA)

STA allows for a multiplexed preamplification by using a pool of gene expression assays as the source of the primers. By using the same assays in the preamplification reaction as the real-time PCR reaction, only targets of interest are amplified. Refer to the *Fluidigm Gene Expression Specific Target Amplification Quick Reference* (PN 68000133) for more information about performing STA.

Preparing 10X Assays

1 In a DNA-free hood, prepare aliquots of 10X assays using volumes in the table below (scale up appropriately for multiple runs).

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Volume per 50 µL Stock
20X TaqMan Gene Expression Assay (Applied BioSystems)	2.5	3.0	25
2X Assay Loading Reagent (Fluidigm, PN 85000736)	2.5	3.0	25
Total Volume	**5.0**	**6.0**	**50**
Final Concentration at 10X	**Primers: 9 µM Probe: 2.5 µM**		

Preparing Sample Pre-Mix and Samples

1 Prepare a Sample Pre-Mix solution containing the Master Mix and 20X GE Sample Loading Reagent sufficient for the number and type of chips to be run.

The following table provides the component amounts for one 48.48 or one 96.96 chip.

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Sample Pre-Mix for 48.48 (µL) (60 for ease of pipetting)	Sample Pre-Mix for 96.96 (µL) (120 for ease of pipetting)
2X Master Mix*	2.5	3.0	180.0	360.0
20X GE Sample Loading Reagent (Fluidigm, PN 85000746)	0.25	0.3	18.0	36.0
cDNA	2.25	2.7		
Total	**5**	**6**		

***** Quanta PerfeCTa® qPCR Fast Mix®, low ROX™ (Quanta BioSciences, PN 95078-012 or VWR, PN 101419-220) or TaqMan Fast Universal PCR Master Mix (Applied Biosystems, PN 4352042) or TaqMan GTXpress Master Mix (Applied Biosystems, PN 4401892) or TaqMan Fast Advanced Master Mix (Applied Biosystems, PN 4444557)

These volumes include some overage to account for pipetting error.

2 In a DNA-free hood, combine the two Sample Pre-Mix components in a 1.5 mL sterile tube--enough volume to fill an entire chip. Aliquot 3.3 µL of the Sample Pre-Mix for each sample.

3 Remove the aliquots from the DNA-free hood and add 2.7 µL of cDNA to each, to make a total volume of 6 µL in each aliquot.

Priming and Loading the Dynamic Array IFC

For instructions on loading the 48.48 Dynamic Array IFC, see *Fluidigm 48.48 Fast Real-Time PCR Workflow Quick Reference* (PN 100-2637). For instructions on loading the 96.96 Dynamic Array IFC, see the *Fluidigm 96.96 Real-Time PCR Workflow Quick Reference* (PN 68000130).

Using the Data Collection Software

The protocols used for data collection are fast protocols.

- **48.48:** Select **GE 48X48 Fast v1.pcl** in the GE folder.
  This cycling protocol is 95°C for 1 minute, followed by 35 cycles of 96°C for 5 seconds and 60°C for 20 seconds. This protocol takes approximately 26 minutes.

- **96.96:** Select **GE 96X96 Fast v1.pcl** in the GE folder.
  The cycling protocol portion of this is the same as for the 48.48 but also includes the Hot Mix protocol for the 96.96 Dynamic Array IFC. The total program runs approximately 66 minutes. The complete program is as follows: 70°C for 30 minutes, 25°C for 10 minutes, 95°C for 1 minute, followed by 35 cycles of 96°C for 5 seconds and 60°C for 20 seconds.

Using UNG for Preventing Carryover Contamination

The Quanta PerfeCTa qPCR Fast Mix and the TaqMan Fast Advanced Master Mix both contain UNG. If using these master mixes, the cycling program can be modified to include a UNG step to protect against carryover contamination. Note the following:

- For the Quanta PerfeCTa qPCR Fast Mix, a 2-minute incubation at 45°C is recommended by the manufacturer. For the 48.48 Dynamic Array IFC, this should be added at the beginning of the program. For the 96.96 Dynamic Array IFC, this should be added before the Hot Mix step.
- For the TaqMan Fast Advanced Master Mix, a 2-minute incubation at 50°C is recommended. This should be added at the beginning of the program for the 48.48 Dynamic Array IFC and before the Hot Mix step for the 96.96 Dynamic Array IFC.

The TaqMan Fast Universal PCR Master Mix contains dUTP and can be modified by the addition of UNG. The TaqMan GTXpress Master Mix was designed for genotyping applications and does not contain any dUTP.

Single-Cell Fast TaqMan Gene Expression Real-Time PCR Using Dynamic Array IFCs

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Introduction

This protocol is intended to be used for fast gene expression analysis of single cells using TaqMan™ Gene Expression Assays on the BioMark™ HD System. The protocol includes three sections: 1) single cell sorting; 2) reverse transcription and specific target amplification (RT-STA); and 3) real-time PCR on either 48.48 or 96.96 Dynamic Array™ integrated fluidic circuits (IFCs). Individual cells are sorted by Fluorescence Activated Cell Sorting (FACS) into a 96-well PCR plate. RT-STA is carried out on a 96-well thermal cycler using the CellsDirect™ One-Step qRT-PCR kit and gene-specific primers included in the TaqMan assays. This reaction generates sufficient template cDNA for TaqMan real-time analysis on Dynamic Array IFCs of hundreds of genes from hundreds of single cells in parallel. Fast real-time PCR for gene expression analysis requires a BioMark HD System, which includes a thermal cycler with fast cycling capabilities. Quanta PerfeCTa™ qPCR Fast Mix from Quanta Biosciences (also available from VWR) is used in combination with TaqMan Gene Expression Assays from Life Technologies Corporation for real-time PCR analysis.

This protocol has been verified on both 48.48 and 96.96 Dynamic Array IFCs.

Required Reagents

- CellsDirect One-Step qRT-PCR Kit (Invitrogen, catalog numbers 11753-100 and 11753-500)
- SUPERase-In (Ambion, PN AM2694)
- DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)
- 20X TaqMan Gene Expression Assays (Applied Biosystems)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)
- Quanta PerfeCTa qPCR Fast Mix, low ROX (Quanta Biosciences, PN 95078-012 or VWR, PN 1014190-220)
- 20X GE Sample Loading Reagent (Fluidigm, PN 85000746)
- PCR certified water (TEKnova, PN W3330)

Required Equipment

- FACS instrument
- Standard 96-well Thermal Cycler
- 96-well PCR plates that are compatible with the FACS instrument and thermal cycler
- Adhesive plate seals (ABI, PN 4311971)
- IFC Controller MX (for the 48.48 Dynamic Array IFC) or HX (for the 96.96 Dynamic Array IFC)
- BioMark HD System

Software Requirements

Fluidigm® Real-Time PCR Analysis Software v.3.0.2 or higher and BioMark HD Data Collection Software v.3.0.2 or higher is required for this protocol.

Cell Sorting Procedure

1. For each 96-well PCR plate, prepare 2X reaction reagent by mixing 580 µL of the CellsDirect 2X Reaction Mix with 11.6 µL of Ambion SUPERase-In.
2. Pipette 5.1 µL of the 2X reaction reagent into each well of the PCR plate and seal the plate with adhesive film. Store the plate on ice if cells are sorted within one day. Otherwise, store at -20°C.
3. Using a FACS instrument, sort cells of interest directly into the plate containing the 2X reaction reagent.
4. Seal plates with adhesive film, vortex for 10 seconds, and centrifuge at 1500 RPM for 1 minute.
5. Use immediately or store at -80°C.

NOTE: 1) The FACS instrument needs to be carefully calibrated to deposit single cells in the center of each well of the PCR plate. 2) Sort in batch mode using a FACS machine. 3) Sort cells into the same plate that will be used for thermal cycling.

Reverse Transcription-Specific Target Amplification (RT-STA)

This reverse transcription preamplification procedure works for both standard and fast TaqMan gene expression applications.

1. In a DNA-free hood, pool all TaqMan Gene Expression Assays and dilute with DNA Suspension Buffer so that each assay is at a final concentration of 0.2X. For example, pipette 4 µL of each of the 96 TaqMan Gene Expression Assays (384 µL total) into a 1.5 ml sterile tube and add 16 µL of DNA Suspension Buffer, resulting in 400 uL of 0.2X primer/probe mix.

2 For each 96-well PCR plate containing sorted cells, prepare the reaction mix by combining 300 µL of the 0.2X Primer/Probe mix, 24 µL of SuperScript III RT/Platinum® Taq Mix and 144 µL of PCR certified water into a 1.5 ml sterile tube. Vortex for 10 seconds.

Component	Volume per Well (µL)	Volume per 96-well Plate with Overage (µL)
0.2X Primer/Probe Mix	2.5	300.0
SuperScript™ III RT/Platinum® Taq Mix	0.2	24.0
PCR certified water	1.2	144.0
Total	3.9	468.0

Table 1 Reaction Mix

3 To each tube of an 8-well PCR strip, add 50 µL of the reaction mix. Transfer 3.9 µL of the reaction mix to each well of the PCR plate containing cells from the strip using an 8-channel pipette, seal, vortex for 10 seconds and centrifuge at 1500 RPM for 1 minute. Place the plate onto a 96-well thermal cycler and proceed to RT-STA using the thermal cycling protocol below:

			STA - 18 Cycles	
	RT	Taq Activation	Denaturation	Annealing/Extension
Temperature	50°C	95°C	95°C	60°C
Time	15 min	2 min	15 sec	4 min

Table 2 Thermal cycle conditions

4 Dilute the resulting cDNA product *1:5* with DNA Suspension Buffer.

Preparing 10X Assays

1 In a DNA-free hood, prepare aliquots of 10X assays using volumes in the table below (scale up appropriately for multiple runs).

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Volume per 50 µL Stock
20X TaqMan Gene Expression Assay (Applied BioSystems)	2.5	3.0	25.0
2X Assay Loading Reagent	2.5	3.0	25.0
Total Volume	5.0	6.0	50.0
Final Concentration at 10X	Primers: 9 µM Probe: 2.5 µM		

Table 3 Assay preparation

Preparing Sample Pre-Mix and Samples

1 Prepare a Sample Pre-Mix solution containing the Master Mix and 20X GE Sample Loading Reagent sufficient for the number and type of chips to be run.

The following table provides the component amounts for one (1) 48.48 or one (1) 96.96 chip.

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Sample Pre-Mix for 48.48 (µL)	Sample Pre-Mix for 96.96 (µL)
(A) 2X Master Mix*	2.5	3.0	180	360
(B) 20X GE Sample Loading Reagent	0.25	0.3	18	36
Diluted RT-STA Sample	2.25	2.7		
Total Volume	5.0	6.0		

*Quanta PerfeCTa™ qPCR Fast Mix, low ROX (Quanta BioSciences, PN 95078-012 or VWR, PN 1014190-220)

Table 4 Sample Pre-Mix and Samples

These volumes include some overage to account for pipetting error.

1 In a DNA-free hood, combine the two Sample Pre-Mix components (A) and (B) from the table above in a 1.5 mL sterile tube--enough volume to fill an entire chip. Aliquot 3.3 µL of the Sample Pre-Mix for each sample to be analyzed in an empty 96-well PCR plate.

2 Remove the Sample Pre-Mix aliquots from the DNA-free hood and add 2.7 µL of the diluted RT-STA sample to each, to make a total volume of 6 µL, then seal, vortex for 10 seconds and centrifuge at 1500 RPM for 1 minute.

Priming and Loading the Dynamic Array IFC

For instructions on loading the 48.48 Dynamic Array IFC, see *Fluidigm 48.48 Real-Time PCR Workflow Quick Reference* (PN 68000089). For instructions on loading the 96.96 Dynamic Array IFC, see *Fluidigm 96.96 Real-Time PCR Workflow Quick Reference* (PN 68000130).

Using the Data Collection Software

The protocols used for data collection are fast protocols.

- **48.48**: Select **GE 48X48 Fast v1.pcl** in the GE folder.

This protocol takes approximately 26 minutes. This cycling protocol is described below:

		Amplification - 35 Cycles	
	Taq Activation	**Denaturation**	**Annealing/Extension**
Temperature	95°C	96°C	60°C
Time	1 min	5 sec	20 sec

Table 5 48.48 protocol

- **96.96**: Select **GE 96X96 Fast v1.pcl** in the GE folder.

The cycling protocol portion of this is the same as for the 48.48, but also includes the Thermal Mix protocol for the 96.96 Dynamic Array IFC. The total program runs approximately 66 minutes. The complete program is described below:

				Amplification - 35 Cycles	
	Thermal Mix		**Taq Activation**	**Denaturation**	**Annealing/ Extension**
Temperature	70°C	25°C	95°C	96°C	60°C
Time	30 min	10 min	1 min	5 sec	20 sec

Table 6 96.96 protocol

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